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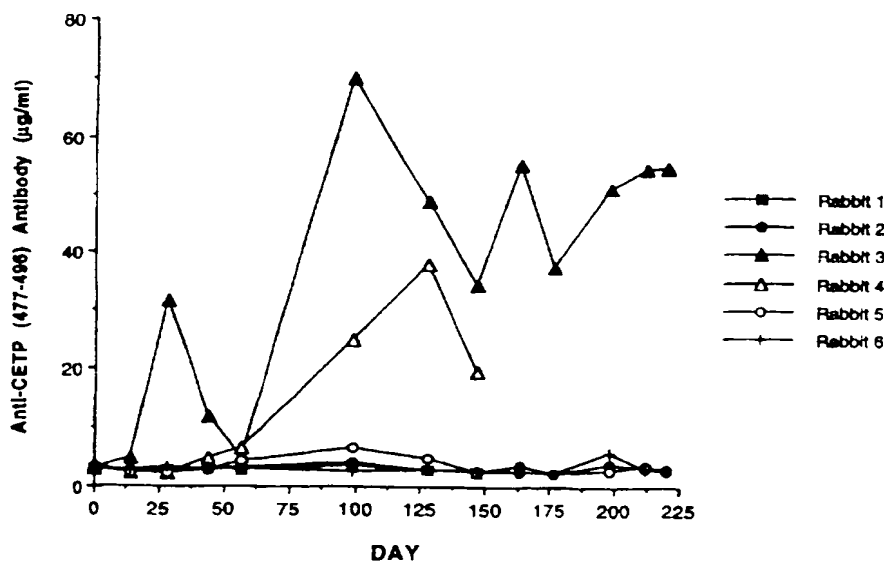
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/12, A61K 31/70		A1	(11) International Publication Number: WO 97/41227
			(43) International Publication Date: 6 November 1997 (06.11.97)
(21) International Application Number: PCT/US97/07294		(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 1 May 1997 (01.05.97)			
(30) Priority Data: 08/640,713 1 May 1996 (01.05.96) US 08/802,967 21 February 1997 (21.02.97) US			
(60) Parent Application or Grant (63) Related by Continuation US 08/802,967 (CIP) Filed on 21 February 1997 (21.02.97)		Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.	
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(54) Title: PLASMID-BASED VACCINE FOR TREATING ATHEROSCLEROSIS



(57) Abstract

A plasmid-based vaccine is provided herein based on the combination of DNA segments coding for one or more B cell epitopes of CETP and one or more broad range helper T cell epitopes. Administration of the plasmids as a vaccine to a vertebrate subject provides an immune response to the subject's endogenous CETP and modulation of CETP activity, leading to prevention or reversal of various manifestations of heart disease. The vaccines provide an advantageous strategy for the prevention or treatment of atherosclerosis.

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Plasmid-Based Vaccine For Treating Atherosclerosis

Cross-Reference to Related Application

This application is a continuation-in-part application of United States Patent Application Serial No. 08/802,967, filed 21 February 1997, which is a continuation-in-part
5 application of United States Patent Application Serial No. 08/640,713, filed May 1, 1996.

Field of the Invention

The present invention relates to the field of immunobiology and specifically to a plasmid DNA vaccine for controlling the activity or effect of cholesteryl ester transfer protein, or CETP, in the body.

Background of the Invention

Cholesterol circulates through the body predominantly as components of lipoprotein particles (lipoproteins), which are composed of a protein portion consisting of one or more apolipoproteins (Apo) and various lipids, including phospholipids, triacylglycerols (triglycerides), cholesterol and cholesteryl esters. There are ten major classes of
15 apolipoproteins: Apo A-I, Apo A-II, Apo IV, Apo B-48, Apo B-100, Apo C-I, Apo C-II, Apo C-III, Apo D, and Apo E.

Lipoproteins are classified by density and composition. High density lipoproteins (HDL), one function of which is to mediate transport of cholesterol from peripheral tissues to the liver, have a density usually in the range of approximately 1.063 - 1.21 g/ml. HDL
20 contain various amounts of Apo A-I, Apo A-II, Apo C-I, Apo C-II, Apo C-III, Apo D, Apo E, as well as various amounts of lipids, such as cholesterol, cholesteryl esters, phospholipids, and triglycerides.

In contrast to HDL, low density lipoproteins (LDL), which generally have a density of approximately 1.019 - 1.063 g/ml, contain Apo B-100 in association with various lipids. In
25 particular, the amounts of the lipids, cholesterol, and cholesteryl esters are considerably higher in LDL than in HDL, when measured as a percentage of dry mass. LDL are particularly important in delivering cholesterol to peripheral tissues.

Very low density lipoproteins (VLDL) have a density of approximately 0.95 - 1.006 g/ml and also differ in composition from other classes of lipoproteins, both in their protein
30 and lipid content. VLDL generally have a much higher amount of triglycerides than do HDL

or LDL and are particularly important in delivering endogenously synthesized triglycerides from liver to adipose and other tissues.

Even less dense than LDL, chylomicrons (density usually less than 0.95 g/ml) contain Apo A-I, Apo A-II, Apo B, Apo C-I, Apo C-II, and Apo C-III and mediate transport of dietary triglycerides and cholesteryl esters from the intestine to adipose tissue and the liver.

The features and functions of the various lipoproteins have been extensively studied. (See, for example, Mathews, C.K. and van Holde, K.E., Biochemistry, pp. 574 - 576, 626 - 630 (The Benjamin/Cummings Publishing Co., Redwood City, California, 1990); Havel, R.J., et al., "Introduction: Structure and metabolism of plasma lipoproteins", in The Metabolic Basis of Inherited Disease, 6th ed., pp. 1129 - 1138 (Scriber, C.R., et al., eds.) (McGraw-Hill, Inc., New York, 1989); Zannis, V.I., et al., "Genetic mutations affecting human lipoproteins, their receptors, and their enzymes", in Advances in Human Genetics, Vol. 21, pp. 145 - 319 (Plenum Press, New York, 1993)).

Decreased susceptibility to cardiovascular disease, such as atherosclerosis, has been generally correlated with increased absolute levels of circulating HDL and also with increased levels of HDL relative to circulating levels of lower density lipoproteins such as VLDL and LDL (see, for example, Gordon, D.J., et al., *N. Engl. J. Med.*, 321: 1311 - 1316 (1989); Castelli, W.P., et al., *J. Am. Med. Assoc.*, 256: 2835 - 2838 (1986); Miller, N.E., et al., *Am. Heart J.*, 113: 589 - 597 (1987); Tall, A.R., *J. Clin. Invest.*, 89: 379 - 384 (1990); Tall, A.R., *J. Internal Med.*, 237: 5 - 12 (1995)).

Cholesteryl ester transfer protein (CETP) mediates the transfer of cholesteryl esters from HDL to triglyceride-rich lipoproteins such as VLDL and LDL, and also the reciprocal exchange of triglycerides from VLDL to HDL (Tall, A.R., *J. Internal Med.*, 237: 5 - 12 (1995); Tall, A.R., *J. Lipid Res.*, 34: 1255 - 1274 (1993); Hesler, C.B., et al., *J. Biol. Chem.*, 262: 2275 - 2282 (1987); Quig, D.W. et al., *Ann. Rev. Nutr.*, 10: 169 - 193 (1990)). CETP may play a role in modulating the levels of cholesteryl esters and triglycerides associated with various classes of lipoproteins. A high CETP cholesteryl ester transfer activity has been correlated with increased levels of LDL-associated cholesterol and VLDL-associated cholesterol, which in turn are correlated with increased risk of cardiovascular disease (see, for example, Tato, F., et al., *Arterioscler. Thromb. Vascular Biol.*, 15: 112 - 120 (1995)).

Hereinafter, LDL-C will be used to refer to total cholesterol, including cholesteryl esters and/or unesterified cholesterol, associated with low density lipoprotein. VLDL-C will be used to refer to total cholesterol, including cholesteryl esters and/or unesterified

cholesterol, associated with very low density lipoprotein. HDL-C will be used to refer to total cholesterol, including cholesteryl esters and/or unesterified cholesterol, associated with high density lipoprotein.

5 All lipoproteins contain apolipoproteins that serve to maintain the structural integrity of lipoproteins and mediate the transport and metabolism of lipids by acting as ligands for specific receptors or co-factors of certain enzymes. In addition to CETP, other proteins, including hepatic lipase, lipoprotein lipase, lecithin:cholesterol acyltransferase (LCAT), LDL receptor, HDL-receptor (SR-B1) and chylomicron remnant receptor, are important in lipid transport and metabolism. Disruption in the function of these components may lead to
10 dyslipidemia, the abnormal metabolism of plasma lipids, which in turn may contribute to the development of atherosclerosis.

The proteins, apolipoproteins, and lipoproteins described above participate in three pathways of lipid transport and metabolism: (1) the chylomicron pathway, (2) the VLDL-LDL pathway, and, (3) the reverse cholesterol pathway. Chylomicrons and chylomicron
15 remnants transport dietary lipids from intestine to peripheral tissues, such as adipose tissue, and the liver. The VLDL-LDL pathway transports lipids from the intestine to peripheral tissues. In the reverse cholesterol pathway excess cholesterol, which cannot be degraded by most tissue, is esterified and delivered either directly in HDL or indirectly after exchange into other lipoprotein fractions to the liver for excretion from peripheral tissues. Specifically,
20 nascent HDL, which is produced by the liver and intestine, enlarges and is transformed into HDL3 and then to HDL2 as cholesterol is acquired and esterified to cholesteryl ester. Cholesteryl esters (CE) can remain with HDL2 for transport and uptake by the liver or can be transferred to lower density lipoproteins, such as VLDL and LDL, by CETP in exchange for triglycerides. In the liver, HDL2 is depleted of triglycerides by hepatic lipase which converts
25 HDL2 back to HDL3 for re-use. During this process CE may also be transferred to hepatocytes. In addition, some HDL may be directly taken up by hepatocytes (see, for example, Havel, R.J., et al., The Metabolic Basis of Inherited Disease, 6th ed., pages 1129 - 1138 (Scriver, C.R., et al., eds.) (McGraw-Hill, Inc., New York, 1989); Fielding, C.J., et al., *J. Lipid Res.*, 36: 211 - 228 (1995)).

30 Thus, the transfer of CE follows one of two pathways. First, lipoproteins may deliver cholesteryl esters to the liver for excretion, thus participating in the reverse cholesterol transport pathway. Second, cholesteryl esters may be recycled back to peripheral tissues.

When all components of these pathways are operating properly, dietary lipids are

rapidly absorbed, transported, and stored or utilized. In the fasting state, lipids are efficiently transported to tissue, and cholesterol is recycled or excreted. Naturally occurring dyslipidemias, perhaps as a result of mutations of apolipoproteins, are often due to dysfunction of one or several of the components in the pathways described above (see, for example, Farmer, J.A. et al., Heart Disease. A Textbook of Cardiovascular Medicine, 4th ed., pp. 1125 - 1160 (Braunwald, E., ed.) (W.B. Saunders Co., Philadelphia, 1992); Havel, R.J., et al, 1992; Zannis, V.I., et al, 1993). Chronic dietary excess of cholesterol may overwhelm normal mechanisms of cholesterol clearance from peripheral tissues, and atherosclerosis may result as evidenced by the development of lesions and blockage of blood flow in cardiovascular tissue.

A number of *in vivo* studies utilizing animal models or humans have indicated that CETP activity can affect the level of circulating cholesterol-containing HDL. Increased CETP-mediated cholesteryl ester transfer activity can produce a decrease in HDL-C levels relative to LDL-C and/or VLDL-C levels, which in turn is correlated with an increased susceptibility to atherosclerosis. For instance, injection of partially purified human CETP into rats (which normally lack CETP activity), was shown to result in a shift of cholesteryl ester from HDL to VLDL, consistent with CETP-promoted transfer of CE from HDL to VLDL (see, Ha, Y.C., et al., *Biochem. Biophys. Acta*, 833: 203 - 211 (1985); Ha, Y.C., et al., *Comp. Biochem. Physiol.*, 83B: 463 - 466 (1986); Gavish, D., et al., *J. Lipid Res.*, 28: 257-267 (1987)). In addition, transgenic mice expressing human CETP were reported to exhibit a significant decrease in the level of cholesterol associated with HDL (see, for example, Hayek, T., et al., *J. Clin. Invest.*, 90: 505 - 510 (1992); Breslow, J.L., et al., *Proc. Natl. Acad. Sci. USA*, 90: 8314 - 8318 (1993)). Furthermore, whereas wild-type mice are normally highly resistant to atherosclerosis (Breslow, J.L., et al., *Proc. Natl. Acad. Sci. USA*, 90: 8314 - 8318 (1993)), transgenic mice expressing a simian CETP were reported to have an altered distribution of cholesterol associated with lipoproteins, namely, elevated levels of LDL-C and VLDL-C and decreased levels of HDL-C (Marotti, K.R., et al., *Nature*, 364: 73 - 75 (1993)). Such transgenic mice expressing simian CETP also were more susceptible to dietary-induced severe atherosclerosis compared to non-expressing control mice and developed lesions in their aortas which were significantly larger in area than found in control animals and more typical of those found in atherosclerosis (Marotti et al., 1993). Intravenous infusion of anti-human CETP monoclonal antibodies (Mab) into hamsters and rabbits inhibited CETP activity *in vivo* and resulted in significantly increased levels of HDL-C levels,

decreased levels of HDL-triglycerides, and increased HDL size, again implicating a critical role for CETP in the distribution of cholesterol in circulating lipoproteins (see, Gaynor, B.J., et al., *Atherosclerosis*, 110: 101 - 109 (1994) (hamsters); Whitlock, M.E., et al., *J. Clin. Invest.*, 84: 129 - 137 (1989) (rabbits)).

5 The role of CETP activity has also been studied in humans. For example, in certain familial studies in Japan, individuals that were homozygous for non-functional alleles of the CETP gene had no detectable CETP activity. Virtually no atherosclerotic plaques were exhibited by these individuals, who also showed a trend toward longevity in their families (see, for example, Brown, M.L., et al., *Nature*, 342: 448 - 451 (1989); Inazu, A., et al., *New Engl. J. Med.*, 323: 1234 - 1238 (1990); Bisgaier, C.L., et al., *J. Lipid Res.*, 32: 21 - 23
10 (1991)). Such homozygous CETP-deficient individuals also were shown to have an anti-atherogenic lipoprotein profile as evidenced by elevated levels of circulating HDL rich in cholesteryl ester, as well as overall elevated levels of HDL, and exceptionally large HDL, i.e., up to four to six times the size of normal HDL (Brown, M.L., et al., 1989, *supra* at p. 451).

15 The above studies indicate that CETP plays a major role in transferring cholesteryl ester from HDL to VLDL and LDL, thereby altering the relative profile of circulating lipoproteins to one that is associated with an increased risk of cardiovascular disease (i.e., decreased levels of HDL-C and increased levels of VLDL-C and LDL-C). Marotti et al. (*Nature*, 364: 73 - 75 (1993)) interpreted their data as indicating that a CETP-induced
20 alteration in cholesterol distribution was the principal reason that arterial lesions developed more rapidly in transgenic, CETP-expressing mice than in non-transgenic control mice when both groups were fed an atherogenic diet.

 CETP isolated from human plasma is a hydrophobic glycoprotein having 476 amino acids and a relative molecular weight of approximately 66,000 to 74,000 daltons on sodium
25 dodecyl sulfate (SDS)-polyacrylamide gels (Albers, J.J., et al., *Arteriosclerosis*, 4: 49 - 58 (1984); Hesler, C.B., et al., *J. Biol. Chem.*, 262: 2275 - 2282 (1987); Jarnagin, S.S., et al., *Proc. Natl. Acad. Sci. USA*, 84: 1854 - 1857 (1987)). A cDNA encoding human CETP has been cloned and sequenced (Drayna, D., et al., *Nature*, 327: 632 - 634 (1987)). CETP has been shown to bind cholesteryl esters (CE), triglycerides (TG), phospholipids (Barter, P.J. et
30 al., *J. Lipid Res.*, 21:238 - 249 (1980)), and lipoproteins (see, for example, Swenson, T.L., et al., *J. Biol. Chem.*, 264: 14318 - 14326 (1989)). More recently, the region of CETP defined by the carboxyl terminal 26 amino acids, and in particular amino acids 470 to 475, has been shown to be especially important for neutral lipid binding involved in neutral lipid transfer

(Hesler, C.B., et al., *J. Biol. Chem.*, 263: 5020 - 5023 (1988)), but not phospholipid binding (see, Wang, S., et al., *J. Biol. Chem.*, 267: 17487 - 17490 (1992); Wang, S., et al., *J. Biol. Chem.*, 270: 612 - 618 (1995)).

5 It follows from current research that increased levels of CETP activity may be predictive of increased risk of cardiovascular disease. Endogenous CETP activity is thus an attractive therapeutic target for modulating the relative levels of lipoproteins to prevent or inhibit the development of or to promote regression of cardiovascular diseases such as atherosclerosis.

10 It would be useful, therefore, to develop the means and methods to control or modulate endogenous CETP activity to prevent or treat cardiovascular disease. Preferably, the modulation of endogenous CETP activity in a human or animal would be accomplished by administering to the subject a pharmaceutical composition that is specific for CETP, does not require large quantities, does not require continuous or frequently repeated dosing, and also does not produce untoward side effects.

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Summary of the Invention

A DNA plasmid-based vaccine is described that comprises a plasmid DNA molecule containing a DNA sequence encoding an immunogenic fusion polypeptide that, when administered to a human or animal subject, will induce the production of autoantibodies specifically reactive with endogenous CETP. Such antibodies inhibit endogenous CETP activity or remove CETP from circulation (clearance), promote the formation and maintenance of an anti-atherogenic serum lipoprotein profile (for example, increased HDL levels and decreased LDL levels), and/or inhibit the development of atherosclerotic lesions.

20 The immunogenic fusion polypeptide encoded on a plasmid as described herein comprises a T cell epitope portion and a B cell epitope portion. A T cell epitope portion encoded on the plasmid of this invention comprises a non-endogenous CETP protein, or fragment thereof, that contains a broad range or "universal" helper T cell epitope which binds the antigen presenting site of multiple (i.e., 2, 3, 4, 5, 6 or more) class II major histocompatibility (MHC) molecules and can form a tertiary complex with a T cell antigen receptor, i.e., MHC:antigen:T cell antigen receptor. By "non-endogenous CETP protein" is meant a protein which is not the endogenous CETP of the individual who is to be administered a plasmid of this invention. Such non-endogenous CETP proteins, or fragments thereof, useful as T cell epitope portions of the immunogenic fusion polypeptide encoded by

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plasmids of this invention include tetanus toxoid (particularly peptides of tetanus toxoid having amino acid sequences of amino acids 2 - 15 of SEQ ID NO:7 and amino acid sequence of SEQ ID NO:10); diphtheria toxin (particularly peptides having amino acid sequences of amino acids 271 - 290, 321 - 340, 331 - 350, 351 - 370, 411 - 430, and 431 - 450 of SEQ ID NO:9); class II MHC-associated invariant chain; influenza hemagglutinin T cell epitope; keyhole limpet hemocyanin (KLH); a protein from known vaccines including pertussis vaccine, the Bacille Calmette-Guerin (BCG) tuberculosis vaccine, polio vaccine, measles vaccine, mumps vaccine, rubella vaccine, and purified protein derivative (PPD) of tuberculin; and also synthetic peptides which bind the antigen presenting site of multiple class II histocompatibility molecules, such as those containing natural amino acids described by Alexander et al. (*Immunity*, 1: 751 - 761 (1994)). When attached to a CETP B cell epitope portion, the T cell epitope portion enables the immunogenic fusion polypeptide to break tolerance in order for antibodies to be made that react with endogenous CETP. By "breaking tolerance" is meant forcing an organism to mount an immune response to a protein, such as endogenous CETP, that the organism does not normally find immunogenic.

The B cell epitope portion of an immunogenic fusion polypeptide encoded on a plasmid of this invention comprises the amino acid sequence of the endogenous CETP, or fragment thereof, of the same species as the individual who will be administered the plasmid; the CETP, or fragment thereof, from a species different from the individual who will be administered the plasmid; or a synthetic amino acid sequence which elicits antibodies that bind to endogenous CETP. Such a B cell epitope portion useful in the plasmid-based CETP vaccine of this invention is encoded by a DNA sequence of at least 15 nucleotides in length.

In one embodiment of the invention, a DNA plasmid contains a structural coding sequence for an immunogenic fusion polypeptide wherein the structural coding sequence comprises a DNA sequence encoding a tetanus toxoid polypeptide (such as nucleotides 13 - 54 of SEQ ID NO:5) as the T cell epitope portion linked in the same reading frame with DNA sequences (such as nucleotides 55 - 159 of SEQ ID NO:5) encoding amino acids 350 - 368 and 481 - 496 of the amino acid sequence of mature rabbit CETP (SEQ ID NO:2) as the B cell epitope portion. In a preferred embodiment, a DNA plasmid of this invention encodes a structural coding sequence for an immunogenic fusion polypeptide wherein the structural coding sequence comprises a DNA sequence encoding a tetanus toxoid polypeptide (such as in nucleotides 13 - 54 of SEQ ID NO:5) as the T cell epitope portion of the immunogenic fusion polypeptide linked in the same reading frame with DNA sequences, such as

nucleotides 1045 - 1101 and 1381 - 1428 of SEQ ID NO:3 encoding, respectively, amino acids 349 - 367 and 461 - 476 of the amino acid sequence of mature human CETP (SEQ ID NO:4) as the B cell epitope portion of the immunogenic fusion polypeptide.

5 The immunogenic fusion polypeptides of the invention are expressed from the plasmids of this invention at sufficient levels and for a sufficient period of time to elicit production of autoantibodies that react specifically with endogenous CETP and that serve to decrease or inhibit CETP-mediated atherogenesis as evidenced by an anti-atherogenic serum lipoprotein profile and/or an inhibition in the development of atherosclerotic lesions.

10 Expression of the immunogenic fusion protein is directed by a promoter or promoter/enhancer sequence that can direct efficient transcription in mammalian cells, particularly skeletal muscle cells. Such promoter/enhancer sequences include, but are not limited to, human cytomegalovirus (CMV) promoter/enhancer sequence, adenovirus promoter/enhancer sequence, and β -actin promoter/enhancer sequence.

15 In addition, a plasmid of this invention may or may not encode an amino terminal secretion signal sequence linked to the immunogenic fusion polypeptide. Preferably, a plasmid of this invention encodes an immunogenic fusion polypeptide that does not contain an amino terminal secretion signal sequence.

20 In another preferred embodiment, a plasmid of this invention also includes a poly A signal sequence located 3' to the structural coding sequence of the immunogenic fusion polypeptide.

25 Thus, a preferred plasmid of this invention consists essentially of a promoter/enhancer sequence which is operably linked to a DNA sequence encoding an immunogenic fusion polypeptide comprising a T cell epitope portion and a B cell epitope portion which induces an individual receiving the plasmid to produce an immune response that results in the inhibition of the activity of endogenous CETP.

30 The DNA plasmids of this invention may be administered by any means normally used to administer plasmid-based vaccines to humans or animals, provided the mode of administration results in expression of the immunogenic fusion polypeptide and production of antibodies which specifically react with (i.e., bind) the endogenous CETP. Preferably, the DNA plasmids are administered intramuscularly or intradermally.

Brief Description of the Drawings

Figure 1 is a diagram showing the construction of plasmid pCMV-LUC which contains a luciferase gene the transcription of which is under the control of a CMV promoter and enhancer.

Figure 2 is a bar graph showing luciferase activity measured at different time points in homogenates of quadriceps from mice injected intramuscularly with plasmid constructs having the luciferase gene under the transcriptional control of three different promoters: CMV, β -actin, and adenovirus. Homogenates were prepared from quadriceps from mice on Day 2, 32, or 132 after being injected with one of the plasmid constructs. "PBS Control" refers to homogenates prepared from control mice on Day 2, 32, or 132 after being injected with sterile phosphate buffered saline (PBS).

Figure 3 is a bar graph showing anti-luciferase antibody production in mice injected intramuscularly with the plasmid pCMV-LUC, in plasma blood samples obtained from mice at 31 and 45 days after immunization.

Figure 4 is a diagram showing the construction of plasmid-based vaccine pCMV-CETP/TT.

Figure 5 shows the nucleotide sequence of a DNA insert encoding a tetanus toxoid fragment and two CETP B cell epitopes, as a fusion polypeptide, inserted under the control of the CMV promoter/enhancer in plasmid pCMV-CETP/TT. The corresponding, single-letter abbreviation of the amino acid sequence for the encoded immunogenic fusion polypeptide and the location of NotI restriction endonuclease cleavage sites in the DNA insert are also depicted.

Figure 6 shows an outline of the daily protocol used for testing the plasmid-based vaccine pCMV-CETP/TT in rabbits #1 - #8 and #10 - #14 (indicated as numbers at top of table columns) under differing dietary conditions. The day on which a particular step (row) of the protocol was carried out on a particular rabbit (column) is indicated in each box. Rabbits #1 - #8 were injected with 50 μ g of pCMV-CETP/TT as a plasmid-based CETP vaccine and 50 μ g of pCMV-LUC as an internal control and reporter on indicated days (see rows labeled "CETP Vaccine"). The first injection of the plasmids to rabbits #1 - #8 occurred on Day 0. Rabbits #10 - #14 were control rabbits which did not receive either of the plasmids (negative control animals). Boxes in rows labeled "PRE 1", "PRE 2", or "PRE 3" indicate days (designated as negative numbers in bold and in parentheses) on which blood samples

("prebleeds") were obtained from rabbits prior to the first administration of the plasmids. "PRE 3" also indicates that blood samples were drawn on the same day as and prior to the first injection of plasmids pCMV-CETP/TT and pCMV-CETP/TT DNA into rabbits #1 - #8. Boxes in rows labeled "BLEED 1" - "BLEED 12" indicate those days (in bold) on which blood samples were obtained from rabbits after the first injection of plasmid DNA into rabbits # 1 - #8 on Day 0. Boxes in rows labeled "Tetanus" indicate the day on which a rabbit received an intramuscular injection of an alum-adsorbed vaccine preparation of tetanus toxoid. Boxes in rows labeled "0.25% Chol." and "0.5% Chol." indicate the day on which a particular rabbit was placed on a rabbit chow diet supplemented with 0.25 % (w/w) cholesterol or 0.5 % (w/w) cholesterol, respectively. Boxes containing an "X" indicate that a particular rabbit was either not in the particular protocol step designated by the row or that the animal had been sacrificed. Boxes in rows labeled "Termination" indicate the day on which each rabbit was sacrificed.

Figure 7 is a histogram showing luciferase expression in tissue homogenates taken from the approximate areas of each of three sites in rabbit quadriceps which were injected with both pCMV-LUC and pCMV-CETP/TT plasmids. Luciferase activity is expressed in counts per second. "Normal Rabbit" refers to luciferase activity in tissue homogenate taken from a normal control rabbit that did not receive either plasmid. "Rabbit 8" refers to tissue homogenates prepared from approximate sites of injection of plasmids pCMV-LUC and pCMV-CETP/TT into quadriceps of rabbit #8 which was sacrificed 48 hours after being injected with plasmids on Day 0. "Rabbit 7" refers to tissue homogenates prepared from approximate sites of injection of pCMV-LUC and pCMV-CETP/TT into quadriceps of rabbit #7 which was sacrificed 48 hours after receiving a second injection (boost) of plasmids pCMV-LUC and pCMV-CETP/TT on Day 28.

Figure 8 is a graph showing detection by ELISA of anti-rabbit CETP₄₇₇₋₄₉₆ antibodies in plasma taken on Day 57 from six rabbits (rabbits #1 - #6) vaccinated with plasmid pCMV-CETP/TT. Plasma was assayed from rabbit #1 (filled square), rabbit #2 (filled circle), rabbit #3 (filled triangle), rabbit #4 (open triangle), rabbit #5 (open circle), and rabbit #6 (cross). "NRP" refers to plasma taken from a control rabbit that was not injected with either plasmid pCMV-LUC or plasmid pCMV-CETP/TT (open square).

Figure 9 is a graph showing detection of anti-rabbit CETP₄₇₇₋₄₉₆ antibodies in plasma taken at Day 220 from four rabbits vaccinated with plasmid pCMV-CETP/TT. Plasma was assayed from rabbit #2 (filled circle), rabbit #3 (filled triangle), rabbit #5 (open circle), and

rabbit #6 (cross). "NRP" refers to plasma taken from a control rabbit that was not injected with plasmids pCMV-LUC and pCMV-CETP/TT (open square).

Figure 10 is a graph showing concentration ($\mu\text{g/ml}$) of anti-CETP₄₇₇₋₄₉₆ antibodies in rabbit plasma samples taken as described in Figure 6. Plasma was assayed from rabbit #1 (filled square), rabbit #2 (filled circle), rabbit #3 (filled triangle), rabbit #4 (open triangle), rabbit #5 (open circle), and rabbit #6 (cross).

Detailed Description of the Invention

The instant invention provides a strategy for the inhibition or prevention of cardiovascular disease, such as atherosclerosis, by modulating CETP activity, either by inhibiting CETP activity by antibody binding or clearing CETP activity from the circulatory system (or both). The modulation of endogenous CETP activity is accomplished using a plasmid-based vaccine. The DNA plasmids described herein encode immunogenic fusion polypeptides which when expressed *in vivo* elicit the production of autoantibodies to inhibit and/or clear circulating endogenous CETP activity. The present invention also provides a method for immunizing a vertebrate, such as a human, to elicit an antibody response to its endogenous CETP and thereby modulate CETP activity.

The various designations for lipids, lipoproteins, and apolipoproteins referred to below are the same as described in the Background above. As noted above, CETP plays a significant role in the transport and distribution of CE and TG between lipoproteins HDL and LDL. A decreased CETP activity produces a non-atherogenic lipoprotein profile or decreases the development of atherosclerosis (see, for example, Mabuchi et al., *Acad. Sci.*, 748: 333 - 341 (1995); Inazu et al., *New Eng. J. Med.*, 323: 1234 - 1238 (1990); Gaynor et al., *Artherosclerosis*, 110: 101 - 109 (1994); Whitlock et al., *J. Clin. Invest.*, 84: 129 - 137 (1989)). Conversely, increased CETP activity produces an atherogenic lipoprotein profile and induces atherosclerosis. The overexpression of CETP in transgenic animals decreases HDL levels and accelerates atherosclerosis (Agellon et al., *J. Biol. Chem.*, 266: 10796 - 10801 (1991); Marotti et al., *Nature*, 364: 73 - 75 (1993)), and the administration of CETP to experimental animals can lead to elevated levels of VLDL-C and LDL-C and a relative decrease in the level of HDL-C (Groener et al., *Biochim. Biophys. Acta*, 1002: 93 - 100 (1989); Ha et al., *Biochim. Biophys. Acta*, 833: 203 - 210 (1985)). Thus, inhibition of CETP activity is a desirable clinical outcome that will help prevent atherosclerosis, and inhibition of CETP activity is an appropriate strategy for promoting a physiological state associated with

prevention and treatment of cardiovascular disease.

In the invention described herein, plasmid-based vaccines are provided for producing autoantibodies directed to endogenous CETP. Specifically, DNA plasmids are described which are administered (for example, by intramuscular injection or intradermal ballistic administration) to an individual. The administered DNA plasmids encode and direct the production of immunogenic fusion polypeptides which exhibit one or more broad range or "universal" helper T cell epitopes and also one or more B cell epitopes of CETP. Such immunogenic polypeptides elicit the production of autoantibodies that react specifically with (i.e., bind to) CETP in the individual (endogenous CETP). The production of anti-CETP antibodies promotes a physiological state associated with decreased risk of cardiovascular disease. The beneficial modulation of CETP activity produced by the DNA vaccines is evidenced by a significantly decreased or eliminated CETP activity; by an anti-atherogenic lipoprotein profile (for example, an increase in the level of HDL or HDL-C compared to LDL, LDL-C, VLDL, or VLDL-C); or by an inhibition (including prevention) or decrease in the development of atherosclerotic lesions in cardiovascular tissue, such as the aorta.

Design of DNA Plasmid Vaccine for Modulation of Endogenous CETP

Many small peptides only become antigenic when coupled to larger immunogenic carrier proteins (see, Etlinger, *Immunol. Today*, 13, 52 - 55 (1992)). The carrier protein is understood to provide epitopes recognized by helper T cells. Thus, although self-antigens, such as B cell epitopes of endogenous proteins, are generally not immunogenic, recent evidence has indicated that self-antigens can be made more immunogenic by linking them to one or more epitopes recognized by helper T cells of the host's immune system. Such immunogenic polypeptides containing one or more helper T cell epitopes and B cell epitopes of a particular endogenous protein may elicit production of autoantibodies that specifically react with the particular endogenous protein. For example, a fragment of human chorionic gonadotropin (hCG) has been conjugated to carrier proteins to produce a peptide vaccine to elicit autoantibodies reactive with hCG (see, Ada, G.L., in *Fundamental Immunology*, 3rd ed. W.E. Paul, ed. (Raven Press, Ltd., New York, 1993) pp. 1309 - 1352; Aitken et al., *Brit. Med. Bull.*, 49: 88 - 99 (1993)). This peptide vaccine consisted of a heterospecies dimer of the alpha-subunit of ovine luteinizing hormone and the beta-subunit of hCG conjugated to either of two immunogenic carrier proteins, tetanus toxoid (TT) or diphtheria toxoid (DT) (Talwar et al., *Proc. Natl. Acad. Sci.*, 91: 8532 - 8536 (1994)). In addition, a peptide vaccine including the C-terminal portion of human CETP and a T cell epitope from tetanus toxoid

was shown to elicit an anti-CETP antibody response and to alter CETP activity in rabbits, as described in commonly assigned, copending U.S. application Serial No. 08/432,483, filed May 1, 1995, incorporated herein by reference.

Broad Range T Cell Epitopes

5 The DNA plasmids described herein comprise a DNA sequence encoding an immunogenic fusion polypeptide comprising a T cell epitope portion and a B cell epitope portion. The helper T cell epitope portion (or simply, "T cell epitope portion") encoded on a plasmid of this invention comprises a non-endogenous CETP protein, or fragment thereof, that contains a "universal" or "broad range" T cell epitope which binds antigen presenting
10 sites of multiple (two or more) class II major histocompatibility (MHC) molecules and can form a tertiary complex with a T cell antigen receptor, i.e., MHC:antigen:T cell antigen receptor, which is the functional unit of T cell epitope recognition. Thus, "universal" or broad range T cell epitopes useful in this invention bind the antigen presenting site of multiple class II MHC molecules and serve to activate helper T cells which, in turn, stimulate
15 B cell growth and differentiation, leading to the secretion of specific antibodies. Preferably, a universal or broad range T cell epitope encoded by a plasmid of this invention binds the antigen presenting site of three or more different class II MHC molecules, such as three different allelic class II MHC molecules, found in the human population. More preferably, a universal or broad range T cell epitope encoded on a plasmid of this invention binds the
20 antigen presenting site of four or more different class II MHC molecules. Most preferably, a universal or broad range T cell epitope encoded on a plasmid of this invention binds six or more different class II MHC molecules.

Broad range antigenic helper T cell epitopes are known in the art. These include, for example, epitopes of tetanus toxoid (TT) and diphtheria toxoid (DT) (see, for example,
25 Panina-Bordignon, P., et al., *Eur. J. Immunol.*, 19: 2237 - 2242 (1989) (characterization of universal tetanus toxoid helper T cell epitope peptides); Etlinger, H., *Immunol. Today*, 13: 52 - 55 (1992); Valmori, D., et al., *J. Immunol.*, 149: 717 - 721 (1992) (use of universal TT epitopes in candidate anti-malarial vaccine); Raju et al., *Eur. J. Immunol.*, 25: 3207 - 3214 (1995) (broad range T cell epitopes of DT); Talwar, G.P., et al., *Proc. Natl. Acad. Sci. USA*,
30 91: 8532 - 8536 (1994) (use of TT and DT as universal epitopes in anti-human chorionic gonadotropin vaccine); Talwar, G.P., et al., *Proc. Natl. Acad. Sci. USA*, 91: 8532 - 8536 (1994)).

In addition to TT and DT, other broad range or universal helper T cell epitope

sequences useful in this invention include the universal class II MHC binding T cell epitopes: HA of influenza hemagglutinin, HBVnc, CS, and MT as described in Alexander et al. (*Cell*, 1: 751 - 761 (1994)). Still other T cell epitopes that may be encoded by the plasmids of this invention include those polypeptides derived from antigenic proteins derived from pertussis vaccine, Bacile Calmette-Guerin (BCG), polio vaccine, measles vaccine, mumps vaccine, rubella vaccine, and purified protein derivative (PPD) of tuberculin (see, for example, Etlinger, H., *Immunol. Today*, 13: 52 - 55 (1992)). Synthetic sequences, i.e., that are not derived from a naturally occurring organism, may also be used. Examples of synthetic broad range T cell epitopes are discussed in Alexander et al., *Immunity*, 1: 751 - 761 (1994).

Plasmids of this invention may encode a variety of non-endogenous CETP proteins, or fragments thereof, such as tetanus toxoid, particularly peptides of tetanus toxoid having amino acid sequences of amino acids 2 - 15 of SEQ ID NO:7 (a corresponding nucleotide coding sequence is nucleotides 13 - 54 of SEQ ID NO:5) and amino acid sequence of SEQ ID NO:10. Another source of universal or broad range T cell epitopes useful in the plasmids of this invention is diphtheria toxin, particularly peptides having amino acid sequences of amino acids 271 - 290, 321 - 340, 331 - 350, 351 - 370, 411 - 430, and 431 - 450 of SEQ ID NO:9. An example of corresponding nucleotide sequences encoding these broad range T cell epitopes from diphtheria toxin are nucleotides 811 - 870, 961 - 1020, 991 - 1050, 1051 - 1110, 1231 - 1290, and 1291 - 1350 of SEQ ID NO:8, respectively. Other sources of universal or broad range T cell epitopes that may be encoded on plasmids of this invention include, but are not limited to, class II MHC-associated invariant chain; hemagglutinin; keyhole limpet hemocyanin (KLH); a protein from known vaccines including pertussis vaccine, the Bacile Calmette-Guerin (BCG) tuberculosis vaccine, polio vaccine, measles vaccine, mumps vaccine, rubella vaccine, and purified protein derivative (PPD) of tuberculin; and also synthetic peptides as described by Alexander et al. (1994).

Furthermore, two or more copies of DNA coding for the same or various different universal helper T cell epitopes may be linked to one another to form multiple or multivalent helper T cell epitope portions of the vaccine peptides of this invention. For example, a plasmid of this invention may contain DNA segments encoding a multiple or multivalent helper T cell epitope portion having an amino acid sequence of a TT helper T cell epitope and a DT helper T cell epitope. The T cell epitope portion of the DNA vaccine may be continuous or may have intervening, in-frame segments encoding the B cell epitope portion or (preferably non-antigenic) segments linking the T and/or B cell epitopes.

A large number of possible T cell epitopes could be used as the T cell epitope portion of an immunogenic fusion polypeptide encoded on a plasmid of this invention. A routine methodology can be used to identify such additional broad range T cell epitopes which bind the antigen presenting sites of multiple class II MHC molecules (for example, Raju et al., *Eur. J. Immunol.*, 25: 3207 - 3214 (1995)). In this methodology, broad range T cell epitopes are identified by first obtaining peripheral blood from individuals that have recently been immunized with a protein of interest, i.e., the protein from which the T cell epitope is derived. Alternatively, peripheral blood from individuals not recently immunized can be used. However, T cells from such individuals need to be stimulated with the protein of interest *in vitro* to increase the number of T cells specific for the protein of interest. It is not necessary to know the identity of such a protein of interest to obtain a T cell epitope useful in this invention. It is sufficient if a protein can be isolated and purified, such as by extracting a band of the protein from a polyacrylamide gel after electrophoresis. Peptides of a protein of interest are made, for example, by limited proteolysis, or if the amino acid sequence of the protein is known, by synthesizing by standard methods overlapping polypeptides of at least five, and preferably approximately twenty, amino acids in length. A preferred group of individuals used as a source of peripheral blood for this methodology is a group of individuals who have recently been immunized with a known prophylactic vaccine, such as tetanus, diphtheria, or influenza vaccines, which contain one or more proteins that can be selected as the protein of interest to derive a useful T cell epitope. Each peptide from the protein of interest is individually co-cultured with peripheral blood lymphocytes purified from the peripheral blood of each individual from the group. The antigen presenting cells in each culture will bind certain peptides to their class II MHC molecules and display these on their cell surface. CD4+ T cells in the culture will bind a subset of these class II MHC bound peptides and consequently form the tertiary complex MHC:T cell epitope:T cell antigen receptor necessary to activate T cells and induce proliferation. Proliferation is detected by standard ³H-thymidine incorporation into DNA. Cultures showing proliferation by this assay indicate that the peptide co-cultured with the cells contained a helper T cell epitope. A peptide that stimulates proliferation of peripheral blood lymphocytes from multiple individuals is a candidate broad range T cell epitope useful in this invention. The amino acid sequence of such a peptide can be determined by standard amino acid sequence analysis. A DNA molecule encoding the peptide is prepared which encodes the peptide. If a DNA molecule encoding the peptide is not already available, a DNA sequence can be deduced

using the genetic code and a DNA molecule having a nucleotide sequence encoding the peptide can be synthesized by standard DNA synthetic methods or obtained from a commercial vendor. The DNA molecule is then inserted in the same reading frame as the DNA sequence encoding the B cell epitope portion of the immunogenic fusion protein on a plasmid of this invention (see below).

B Cell Epitopes of CETP

The B cell epitope portion of the immunogenic fusion polypeptide encoded by the DNA plasmids of this invention comprises at least one B cell epitope of CETP, preferably the endogenous CETP of the vertebrate subject to be immunized. The use of at least two B cell epitopes is desirable and preferred because it increases the probability that the various autoantibodies produced in response to expression of the DNA vaccine *in vivo* will be able to bind to at least two distinct epitopes on each CETP molecule and thereby promote formation of immune complexes, which leads to efficient clearing of the CETP protein molecules from circulation.

B cell epitopes useful in this invention may be as small as 5 to 8 consecutive amino acid residues of the entire amino acid sequence of CETP. The DNA plasmids described herein contain a DNA sequence encoding a CETP B cell epitope portion of at least 15, and preferably 30 - 48 nucleotides in length. Preferred B cell epitopes of CETP for use in human vaccines will be encoded, individually, by at least a 15-nucleotide sequence of the coding sequence for CETP (see, for example, SEQ ID NO:1 encoding mature CETP (rabbit); SEQ ID NO:3 encoding mature CETP (human)), or degenerate sequences thereof encoding the same epitope. It should be noted that alleles of the rabbit CETP gene have been discovered and consequently, could be anticipated for the human gene (see, Nagashima et al, *J. Lipid Res.*, 29: 1643 - 1649 (1988); Kotake et al., *J. Lipid Res.*, 37: 599 - 605 (1996)). The B cell epitopes may be present in sequence or separated by intervening, in-frame segments encoding the T cell epitope(s) or (preferably non-antigenic) linking peptides of one or more amino acids. Of course, the actual length of the DNA sequence encoding the B cell epitope portion depends on the length of the particular B cell epitopes selected from CETP.

Although the DNA sequence encoding the B cell epitope portion of the immunogenic fusion polypeptide may encode two or more B cell epitopes of CETP, there are several reasons why the DNA sequence should not encode the entire amino acid sequence of the mature circulating CETP. For example, using less than the entire structural coding sequence for CETP limits the probability of producing antibodies that might cross-react with other self

proteins. In addition, using less than the entire CETP structural coding sequence is one way to avoid producing potentially functional CETP protein or fragment, i.e., that would exhibit CE and/or TG transfer activity and thereby *increase* the overall CETP activity in the vaccinated individual. Using less than the entire CETP coding sequence also reduces the chance of eliciting cell-mediated autoimmune responses. Whether a region of CETP or even a particular CETP B cell epitope also includes a T cell epitope can be readily determined by testing the B cell epitope or region of CETP in a cytotoxic T cell or proliferation assay (see, for example, Current Protocols in Immunology, (Coligan et al., eds.) (John Wiley & Sons, New York, 1994) pp. 3.11.4 - 3.11.7 and 3.12.9 - 3.12.14).

The carboxyl terminal 26 amino acids of human CETP is involved in neutral lipid transfer activity (Swenson et al., *J. Biol. Chem.*, 264: 14318 - 14326 (1989)). In particular, a 13-amino acid sequence (Phe-463 to Leu-475 in human CETP, SEQ ID NO: 4; amino acids Phe-483 to Leu-495 in rabbit CETP, SEQ ID NO:2) and also possibly Asp-460 (human) (Asp-480, rabbit) are particularly important for neutral lipid binding and transfer activity (Wang et al., *J. Biol. Chem.*, 268: 1955 - 1959 (1993); Wang et al., *J. Biol. Chem.*, 267: 17487 - 17490 (1992)). This region has already been shown to be immunogenic as a B cell epitope of CETP, and a monoclonal antibody (TP2) directed at this region has been shown to inhibit neutral lipid transfer. Accordingly, in a preferred embodiment, a plasmid used as a DNA vaccine useful in humans comprises a DNA sequence encoding the CETP B cell epitope as defined by the amino acid sequence of Phe-461 to Ser-476 or Phe-463 to Leu-475 of mature human CETP (see SEQ ID NO: 4).

A DNA sequence encoding a second B cell epitope of CETP is defined by the amino acid sequence of Leu-349 to Ile-367 of human CETP (SEQ ID NO: 4) (corresponding rabbit amino acid sequence Arg-350 to Ile-368 of SEQ ID NO: 2). In preferred embodiments this DNA encoding this epitope is included in the structural coding sequence of the immunogenic polypeptide to produce a second antibody species, specific for a second CETP epitope, *in vivo*. Antibodies to a second epitope would allow the formation of immune complexes involving CETP, and consequently promote the removal (clearance) of the complexed CETP. This peptide was selected for its potential antigenicity and high possibility for surface expression on native CETP.

Other suitable B cell epitopes of CETP could be selected, for example, based on previously defined antibody binding sites (see, for example, Roy et al., *Lipid Res.*, 37: 22 - 34 (1996)) or by analysis of the amino acid sequence for structural motifs associated with a

propensity for antibody recognition.

Transcription and Replication Control Sequences

The DNA plasmids of this invention must contain the DNA sequences necessary to permit a sufficient level of *in vivo* expression of the encoded immunogenic fusion polypeptide to elicit production of autoantibodies reactive with endogenous CETP. Thus, the DNA
5 plasmid according to the present invention comprises: the structural coding sequence for an immunogenic fusion polypeptide comprising a DNA sequence coding for at least one T cell epitope and a DNA sequence coding for at least one B cell epitope of CETP as described above, and a promoter sequence or a promoter/enhancer sequence to direct transcription of
10 the structural coding sequence for the immunogenic fusion polypeptide. In some instances it may be desirable, as described above, to include coding sequences for one or more additional amino acids, for example, to space the epitope portions, to disrupt unintentionally created neo-epitopes formed by the juxtaposition of the selected T and/or B cell epitopes, to insert proteolytic cleavage sites, etc. It may also be desirable to include a bacterial origin of
15 replication and a selectable marker(s), for example, to aid in production of large quantities of the plasmid vaccine in bacterial culture.

Transcription of the immunogenic fusion protein structural coding sequence is under the control of a promoter sequence and an enhancer sequence. A variety of promoter and enhancer sequences are known and may be evaluated for this purpose in accordance with
20 Example 1, below. Promoter/enhancer sequences that may be used in plasmids of this invention include, but are not limited to, CMV promoter/enhancer sequence, adenovirus promoter/enhancer sequence, and β -actin promoter/enhancer sequence. In a preferred embodiment, the promoter and enhancer sequences are the CMV immediate-early promoter/enhancer sequence. Whether a particular promoter/enhancer is more or less useful
25 than another promoter/enhancer sequence in the plasmids of this invention can be determined by comparing the ability of promoter/enhancer evaluated by testing whether the promoter/enhancer permits expression of a standard reporter gene, such as luciferase or β -galactosidase, and the production of antibody reactive with the expressed reporter in an animal model for gene expression, such as in rabbits or mice. Generally the higher the level
30 of expression of the reporter gene product and/or the higher the level of production of antibodies reactive with the expressed reporter gene product, the more useful that particular promoter/enhancer will be at directing transcription of the structural coding sequence for the immunogenic fusion protein in the plasmids used as DNA vaccines.

Methods of Administering DNA Vaccine

The plasmid-based vaccines according to the invention may be administered in any conventional manner. Suitable methods include, for instance, direct administration of plasmid DNA via intramuscular injection, intradermal injection or DNA-coated microprojectiles. The amount of vaccine administered will vary widely according to the method of administration, the tissue (for example, skeletal muscle, skin) into which the vaccine is administered, the desired titer of anti-CETP antibodies, the particular therapeutic needs of the subject to be immunized, etc. Very large amounts of DNA vaccine, on the order of 10 mg/kg of body weight, may be administered with injection into muscle tissue, whereas for coated microprojectiles very much less vaccine may possibly be used. The dosage of vaccine and immunization protocol should be calibrated to obtain a beneficial response, which can be measured in a variety of ways, depending on the clinical setting, for example, by measuring change in lipoprotein profile (for example, increased HDL/LDL ratio), anti-CETP antibody titer, serum CETP concentration, change in CETP activity, etc.

The following examples are provided in order to illustrate the invention described herein. These examples are not intended to in any way limit the scope of the invention.

Example I

Selection of the optimal promoter/enhancer and derivation of the pCMV-LUC plasmid

This experiment was designed to evaluate the effectiveness of several promoter/enhancers to express a reporter gene (luciferase) and elicit immune responses, to select the best one for use in future vaccination experiments.

Three different plasmids were constructed, with the firefly luciferase gene expressed under the control of the β -actin, the adenovirus, or the human cytomegalovirus (CMV) immediate early promoter/enhancer. Since the CMV construct (pCMV-LUC) was used in further experiments, details of its construction are as follows.

The CMV promoter/enhancer, with the pUC19 plasmid vector backbone containing the ampicillin resistance gene (amp^r), was excised by digestion with BamHI from the plasmid pCMV β (Clontech Laboratories, Palo Alto, CA). The luciferase gene (LUC), with adjacent splice donor/acceptor sites and polyadenylation signal derived from SV40, was generated from the pGL2-Promoter Vector (Promega Corp., Madison, WI) on a BamHI fragment, as follows: pGL2 was digested with HindIII and ends were filled in with Klenow polymerase. BamHI linkers were attached and digested with BamHI. This LUC fragment was gel purified and ligated to the CMV+ vector fragment from pCMV β . The structure of the resulting

plasmid, pCMV-LUC, was confirmed by restriction mapping. See Figure 1.

Luciferase expression was confirmed by assaying luciferase activity in lysates of COS cells transfected with all 3 constructs.

Four groups of nine mice were established. Three of the groups were injected intramuscularly, in both quadriceps, with 50 µg/quadriceps of one of the three constructs (above) in 25 µl phosphate buffered saline (PBS). The fourth group of mice served as a control and received two 25 µl injections of PBS only. The animals received an equal boost of the same plasmid (or PBS control) after 4 weeks and were bled at approximately 2-week intervals. One mouse from each group was sacrificed at day 2 and at day 32 (48 hours after injections) in order to assay tissue for luciferase production. At the conclusion of the experiment the animals were euthanized with CO₂ and the injected muscle tissue was assayed for luciferase production.

Quadriceps tissue samples were prepared by mechanical homogenization of the muscle with 400 µl reporter lysis buffer (Promega Corp., Madison, WI). The homogenate was vortexed and centrifuged at high speed and the supernatant removed. One hundred µl of beetle luciferin (Promega Corp.) was added to 20 µl of the supernatant. The light emitted due to the enzyme-substrate interaction was measured for 5 seconds in a Packard Top Count scintillation counter. Active luciferase enzyme was detected in tissue samples from animals injected with all three of the plasmids. However, the animals injected with pCMV-LUC had the highest level of active enzyme production (see, Figure 2), and this promoter/enhancer was selected for use in further experiments. The values for days 2 and 32 involved one animal only. On day 132, when the remaining animals were sacrificed and the quadriceps muscles were assayed, significant active luciferase was detected in the CMV group at levels as high as or higher than that detected on days 2 and 32. It is particularly striking that active luciferase was found in muscle tissue 132 days after the last injection of pCMV-LUC. The longevity of the expression of protein with the pCMV-LUC construct was important to the logic of using the CMV promoter/enhancer for the CETP vaccine plasmids described below.

Antibodies to luciferase were detected in bleeds taken on day 31 and 45. The ELISA was performed as follows: Biotinylated luciferase was adhered to a streptavidin-coated plate for 1 hour, then washed with PBS containing 0.05% Tween 20. Mouse plasma was diluted in PBS with 1% BSA in PBS, incubated in the plate for approximately two hours, then washed. Goat-anti-mouse-HRP (goat anti-mouse antibody conjugated to horseradish peroxidase) was added and incubated for 45 minutes. After incubation for 2 hour at 20°C on a rotating

shaker, and washing, the reaction was developed with 3,3',5,5'-tetramethylbenzidine (TMB, Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD), stopped with 2N H₂SO₄, and read at 450 nm. Antibodies to luciferase were detected in bleeds taken on day 31 and 45. The results are presented graphically in Figure 3.

5 A significant increase in antibody production was seen subsequent to the second injection of plasmid. This experiment indicates that luciferase produced by a plasmid-based vaccine can elicit antigen-specific antibodies. These experiments indicated that the CMV promoter/enhancer is effective at driving the expression of an immunogenic protein *in vivo*.

Example II

10 pCMV-CETP/TT plasmid vaccine design and construction

 The rabbit CETP fragment corresponding to the C-terminal amino acids 481 - 496 (see, SEQ ID NO: 2) has been identified to contain the functional, neutral lipid binding site of rabbit CETP. This fragment includes the epitope recognized by TP2, an anti-CETP monoclonal antibody that inhibits CETP activity (Swenson, T. L., et al., *J. Biol. Chem.*, 264: 15 14318 - 14326 (1989)). A second epitope of rabbit CETP (amino acids 350-368 of SEQ ID NO:2) was selected for the plasmid-based vaccine to elicit antibodies to a second epitope which would allow the formation of immune complexes involving CETP, and consequently promote the clearance of the immune complexed CETP. This epitope was selected for its potential antigenicity and high possibility for surface expression on native CETP.

20 A tetanus toxoid sequence recognized as almost universally antigenic (Panina-Bordignon, P., et al., *Eur. J. Immunol.*, 1989: 2237 - 2242 (1989)) was selected as the T cell epitope portion. This TT epitope has been used successfully in generating an autoimmune antibody response to hCG (Talwar, G. P., et al., *Proc. Natl. Acad. Sci.*, 91: 8532 - 8536 (1994)). It would be expected to be particularly effective also in vaccines administered to 25 subjects previously vaccinated with tetanus toxoid.

 A set of four oligonucleotides were synthesized which encode the TT and CETP epitopes as well as an initiating methionine residue, a 5' Kozak sequence (for efficient translation), a stop codon, and flanking NotI sites for cloning. The oligonucleotides were annealed and extended with DNA polymerase. See Figure 4.

30 The double-stranded product was digested with NotI and gel purified to isolate the CETP/TT insert below:

GCGGCCGCC ATG CAG TAC ATC AAG GCC AAC TCC AAG TTC ATC GGC ATC ACG GAG
 CGCCGGCGC TAC GTC ATG TAG TTC CGG TTG AGG TTC AAG TAG CCG TAG TGC CTC
 NotI M Q Y I K A N S K F I G I T E
 |< TETANUS TOXOID >|

5

CGC TTC CCC CGC CCA GAT GGC CGA GAA GCT GTG GCC TAC AGG TTT GAG GAG GAT ATC
 GCG AAG GGG GCG GGT CTA CCG GCT CTT CGA CAC CGG ATG TCC AAA CTC CTC CTA TAG
 R F P R P D G R E A V A Y R F E E D I
 |< rabbit CETP (350-368) >|

10

TTC GGT TTT CCC AAG CAC CTG CTG GTG GAT TTC CTG CAG AGC CTG AGC TAG CGGCCGC
 AAG CCA AAA GGG TTC GTG GAC GAC CAC CTA AAG GAC GTC TCG GAC TCG ATC GCCGGCG
 F G F P K H L L V D F L Q S L S *
 |< rabbit CETP (481-496) >| STOP NotI

15

In the above insert, the coding strand is SEQ ID NO: 5, the antisense strand is SEQ ID
 NO: 6, the amino acid sequence is SEQ ID NO: 7. The insert is also depicted in Figure 5.

20

The plasmid pCMV β (Clontech Laboratories) was digested with NotI to generate a
 fragment containing the CMV promoter/enhancer on a pUC19 backbone, with the ampicillin
 resistance gene (amp^r). This fragment also includes splice donor/acceptor sites and a
 polyadenylation signal derived from SV40, flanking the NotI insertion site. The synthesized
 CETP/TT insert was ligated to the CMV+ vector fragment from pCMV β . Plasmids were
 recovered by bacterial transformation and inserts confirmed by DNA sequencing.

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Example III

Vaccination of rabbits with the pCMV-LUC and pCMV-CETP/TT plasmids

An experiment employing a rabbit model for atherosclerosis (Daley et al., *Arterioscl.*
Thromb., 14: 95 - 104 (1994)) was designed to test whether a DNA plasmid-based vaccine
 according to this invention would break tolerance to endogenous CETP resulting in
 production of antibodies reactive with endogenous CETP and/or inhibition in the
 development of atherosclerotic lesions in the rabbit aorta. New Zealand white rabbits (n = 8)
 were immunized with both plasmids pCMV-LUC and pCMV-CETP/TT and monitored for
 production of anti-luciferase and anti-CETP antibody production as well as for the ability to
 inhibit progression of atherosclerosis when placed on atherogenic diets, i.e., diets
 supplemented with amounts of cholesterol known to generate definite and extensive
 atherosclerotic lesions in control rabbits (Daley et al., *id.*). The daily protocol for this
 experiment employing 13 rabbits is shown in Figure 6.

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Eight rabbits (rabbits #1- #8) were vaccinated in three sites intramuscularly in each
 quadriceps with a vaccine preparation consisting of an equal mixture of the plasmids pCMV-

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CETP/TT and pCMV-LUC on Day 0. pCMV-LUC served as a reporter plasmid to allow an additional level of experimental quantitation of plasmid-dependent protein expression and of antibody production to the plasmid-encoded, expressed protein. Specifically, blood samples (for example, 3 - 5 ml from an ear vein) were taken from the rabbits once a week for 3 weeks to establish various pre-vaccination values ("prebleeds" designated PRE 1, PRE 2, and PRE 3 in Figure 6). Blood samples, taken after an overnight fast (about 16 hours), were collected into EDTA anti-coagulant. Following the last prebleed (PRE 3), each animal was vaccinated with three injections into each quadriceps. Each of these 6 injections consisted of 50 μ g of the pCMV-LUC plasmid and 50 μ g of the pCMV-CETP/TT plasmid in 100 μ l of PBS containing a small amount of carbon powder (to aid in excising the injection site). Forty-eight hours after the vaccination one rabbit (rabbit #8) was sacrificed, and its blood and quadriceps were removed for analysis. All animals were bled and boosted (as above, except that blue dye was used instead of carbon) four weeks (Day 28) after the primary vaccination. Again, 48 hours after the boost vaccination, one rabbit (rabbit #7) was sacrificed, and its blood and quadriceps were removed.

The tissue samples around the areas of carbon marks in the quadriceps of rabbits #8 and #7, respectively, were taken and tissue homogenates prepared. Using the luciferase assay described above, luciferase enzymatic activity was detected in tissue taken from the primary (Day 0) injection sites as shown in Figure 7. For example, unvaccinated muscle tissue gave a background signal of approximately 5.33 counts per second (cps) in this assay (Normal Rabbit in Figure 7). Muscle tissue from a vaccinated site of rabbit #8 (site 3 for Rabbit 8 in Figure 7), removed 2 days after vaccination, gave a luciferase signal of 125 cps (23.5 times background), and muscle tissue from vaccinated sites of rabbit #7, removed 30 days after vaccination showed a luciferase signal of 26.7 cps (site 1 for Rabbit 7 in Figure 7, 5 times background) and 168 cps (site 3 for Rabbit 7 in Figure 7, 31.5 times background). The fact that not all tissue samples showed luciferase activity was probably due to the difficulty in locating precisely the sites of deposition of plasmid despite the use of carbon to mark the vaccination sites. However, the data from samples from site 3 of rabbits #8 and #7 shown in Figure 7 clearly demonstrate that this plasmid construct is effective as a vaccine vector (regardless of insert) in rabbits.

Two additional blood samples (BLEEDS 3 and 4 on Days 44 and 57, respectively, in Figure 6) were taken at two-week intervals. The animals were then vaccinated three times intramuscularly with 0.5 ml of an alum-adsorbed preparation of tetanus toxoid (Connaught

Laboratories, Inc., Swiftwater, PA) on Days 66, 91, and 128 (see Figure 6). This was done to determine if tetanus vaccination would increase the CETP vaccine efficacy and to better mimic the human situation.

Initially, all rabbits in this experiment were fed standard rabbit chow. In order to induce the formation of atherosclerotic-like lesions, rabbits were placed on a diet containing either 0.25 % (w/w) cholesterol or 0.5 % (w/w) cholesterol at Days 99, 112, and 154, as indicated in Figure 6. Additional blood samples (BLEEDS 8 - 12) were taken, and the entire experiment terminated by Day 220.

An ELISA designed to detect free serum antibodies recognizing a rabbit CETP peptide having an amino acid sequence corresponding to amino acids 477 - 496 of rabbit CETP (amino acids 477 - 496 of SEQ ID NO:2) was performed essentially as follows: Wells of a 96-well streptavidin-coated plate were coated with the CETP 477 - 496 biotinylated peptide by incubation of 100 μ l of a solution of the peptide (1.0 μ g/ml PBS) for 30 minutes to 1 hour, then washed with 2x PBS containing 0.1% Tween 20. Immunized rabbit plasma (or normal rabbit plasma; NRP) was diluted in PBS with 1% bovine serum albumin (BSA), incubated in the plate for approximately two hours, then washed. Goat-anti-rabbit-HRP (goat anti-rabbit antibody conjugated to horseradish peroxidase) was added and incubated for approximately 45 min. on a rotating shaker. Following washing, the reaction was developed with 3,3',5,5'-tetramethylbenzidine (TMB, Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD), stopped with 2N H₂SO₄, and read at 450 nm spectrophotometrically using an ELISA plate reader.

Plasma samples from the six rabbits (rabbits #1 - #6), taken 57 days after the primary vaccination were diluted and assayed for production of antibody reactive with the rabbit CETP₄₇₇₋₄₉₆ peptide. Plasma from an uninjected rabbit (NRP) was also assayed. The results are depicted in Figure 8, indicating a range in the levels of production of anti-rabbit CETP 477 - 496 peptide antibody in vaccinated animals by Day 57.

Plasma samples from rabbits #2, #3, #5, and #6 were also taken on Day 220 and assayed to determine whether rabbits vaccinated with pCMV-CETP/TT continued to produce detectable levels of antibody to CETP as determined by an ELISA using the rabbit CETP 477 - 496 peptide. Wells of a 96-well streptavidin-coated plate were coated with biotinylated CETP₄₇₇₋₄₉₆ peptide by incubation of 100 μ l of a solution of the peptide (200 ng/ml in PBS) for 1 hour. Non-specific binding was prevented by incubating with Blocking Buffer (PBS with 1% (w/v) BSA, 1% (w/v) non-fat dry milk, 0.5% (w/v) gelatin, 0.9% (v/v) Triton X-100,

and 0.6% (v/v) NP-40) overnight at 4°C on rotating shaker at 150 rpm, followed by washing three times with Wash Buffer (2x PBS with 0.05% (v/v) Tween 20). Immunized rabbit plasma (or normal rabbit plasma, NRP) was diluted in Blocking Buffer, incubated in the plate for approximately two hours, then washed. Goat-anti-rabbit-HRP was added and incubated for approximately 1 hour on a rotating shaker. Following washing, the reaction was developed with TMB, stopped with 50 µl 2N H₂SO₄, and read at 450 nm on an ELISA plate reader. Data from the plasma of the four vaccinated rabbits taken on Day 220 and normal rabbit plasma (NRP) are shown in Figure 9.

The data in Figure 9 indicate that plasma from rabbit #3 clearly contained detectable antibody to the rabbit CETP₄₇₇₋₄₉₆ peptide according to this ELISA. Using this ELISA, plasma from rabbit #6 also appeared to contain some detectable anti-CETP antibody. However, while the signal from the plasma of rabbit #3 was substantial, the signal from the plasma of rabbit #6 was near baseline (see, for example, Figure 9). This signal is interpreted as indicating the presence of antibodies in rabbit #3 and probably rabbit #6 recognizing this epitope of CETP.

The plasma samples were assayed in another ELISA designed to quantitate antibody to the CETP₄₇₇₋₄₉₆ peptide. Wells of a 96-well streptavidin-coated plate were coated with biotinylated CETP₄₇₇₋₄₉₆ peptide by incubation of 100 µl of a solution of the peptide (1 µg/ml in PBS) for 30 minutes to overnight, then washed with PBS containing 0.05% (v/v) Tween 20. Non-specific binding was prevented by incubating with Blocking Buffer (described above) for two hours at room temperature on a rotating shaker at 150 rpm, followed by washing four times with Wash Buffer (described above). Immunized rabbit plasma was diluted in Blocking Buffer, incubated in the plate for 1.5 hours, then washed. Goat-anti-rabbit-HRP was added and incubated for approximately 1 hour on a rotating shaker. Following washing, the reaction was developed with 100 µl of TMB, stopped with 50 µl of 2N H₂SO₄, and read at 450 nm in an ELISA plate reader. The concentration of the specific antibodies was estimated using a standard curve made from biotinylated rabbit immunoglobulin at 15 to 250 ng/ml.

The results of this assay are shown in Figure 10. Again, the plasma sample of rabbit #3 clearly contained detectable antibody reactive with the rabbit CETP₄₇₇₋₄₉₆ peptide. However, this assay did not detect antibody reactive with the peptide in plasma samples of rabbits # 2, #5, and #6. Unimmunized rabbits #10, #12, and #13 exhibited a background signal in this assay similar to rabbit #2. The plasma sample of rabbit #4 also appeared to

contain detectable antibody to CETP according to this assay. However, rabbit #4 was terminated according to the protocol at day 148 (see Figure 6), whereas rabbits #2, #3, #5, and #6 were alive throughout the entire 220 days of the experiment.

5 The observation that there was inter-animal variation in the response to the vaccine is consistent with the literature on plasmid-based vaccines. It can consequently be deduced that the plasmid-based vaccine produced the desired protein in a humorally immunogenic form. It is important to note that this assay detects only free antibody in the plasma samples. Anti-CETP antibodies bound to endogenous CETP (presumably the majority) would not be detected. Also, these rabbits had not been previously vaccinated with tetanus vaccine. It is
10 likely that titers would be raised in a subject receiving or having previously received tetanus vaccine, such as is the case for many adult humans who have received vaccinations against tetanus.

As indicated in the daily protocol in Figure 6, rabbits #2, #3, #5 and #6 in this experiment were switched from a diet of basic rabbit chow to diets supplemented with
15 various amounts of cholesterol known to produce atherosclerotic-like lesions in rabbits (Daley et al., *Arterioscler. Thromb.*, 14: 95 - 104 (1994)) on Days 99, 112, and 154. To determine whether the plasmid-based vaccine may affect the development of atherosclerosis, the aortas of these rabbits were examined histologically for the development of atherosclerotic lesions. After blood samples were taken on Day 220, rabbits #2, #3, #5, and
20 #6 were sacrificed. The entire aortas from each of the four rabbits were removed and placed into fixative solution (3.7 % v/v formaldehyde). Loose tissue, adherent fat, and the adventitia were dissected free from the arteries. Each artery was then cut lengthwise, pinned flat to expose the intimal (luminal) surface, stained with Sudan IV, and then photographed. Sudan IV is a fat soluble red dye that stains atherosclerotic plaques on the intimal surface of arteries.
25 The stained aortas of rabbits #2 and #5 revealed a prevalence of atherosclerotic lesions along the length of the aortas and particularly in the portion of the aortas from the thoracic region. The aortas of rabbits #2 and #5 were similar to those of unvaccinated rabbits on a cholesterol-supplemented, atherogenic diet (such as rabbits #10, #12, and #13). In contrast, the aortas of rabbits #3 and #6 had a much smoother and more uniform appearance on the
30 intimal surface owing to a lower incidence of lesions, including the portion of the aorta from the thoracic region.

To quantify the noticeable difference in the presence of atherosclerotic lesions in the aortas of rabbits #2 and #5 and lack thereof in the aortas of rabbits #3 and #6, the surface area

of the pinned aortas and that of the aortic lesions was determined from photographs by planar morphometry (Daley et al., 1994) using a digitizing tablet with associated software (THE MORPHOMETER™, Woods Hole Educational Associates, Woods Hole, Massachusetts). The percentage of the surface area of the aortas covered by lesions was determined to be 44.8 % for rabbit #2, 50.9 % for rabbit #5, 14.2 % for rabbit #3, and 14.4 % for rabbit #6.

In summary, rabbits #2 and #5, did not produce detectable anti-rabbit CETP antibody as determined by ELISA using the rabbit CETP 477 - 496 peptide after 220 days on the vaccination protocol described above and shown in Figure 6, and these rabbits developed significant atherosclerotic lesions over the intimal surface of their aortas (44.8 % and 50.9 %, respectively) after eating a diet supplemented with cholesterol for about 17 weeks. In contrast, rabbits #3 and #6, which probably produced anti-rabbit CETP antibody, had noticeably less surface area of their aortas covered with atherosclerotic lesion (14.2 % and 14.4 %, respectively) after approximately 17 weeks on an elevated cholesterol diet.

Example IV

The results of the above experiment using a rabbit model for atherosclerosis indicate that the plasmid-based vaccines of this invention may be used to prevent or treat atherosclerosis in other vertebrates. By analogy to the treatment for inhibiting atherosclerosis in rabbits illustrated in Example III, similar plasmid constructs may be made for other vertebrates, including humans. Such plasmids encode an immunogenic fusion polypeptide comprising a universal or broad range T cell epitope, such as from tetanus toxoid or diphtheria toxoid, linked in the same reading frame to at least one, more preferably two, B cell epitopes of the endogenous CETP of the individual. An example of a plasmid-based vaccine for endogenous human CETP contains a DNA sequence encoding a translation initiating methionine linked to a TT polypeptide, such as in nucleotides 10 - 54 of SEQ ID NO:5, which is linked in the same reading frame (with or without intervening linker sequences) to a DNA sequence encoding regions of human CETP analogous to those used in the rabbit CETP plasmid-based vaccine, such as nucleotides 1045 - 1101 and 1381 - 1428 of SEQ ID NO:3 encoding amino acids 349 - 367 and 461 - 476 of SEQ ID NO:4, respectively. Preferably, the DNA sequence in the plasmid for use as a vaccine against human endogenous CETP also includes regions as shown in Figure 5, such as translational start and stop codons and flanking restriction endonuclease sites that are commonly employed for plasmid construction and gene expression.

Example V

In another aspect of this invention, plasmid-based vaccines can be made in which a plasmid encodes a universal or broad range T cell epitope portion linked in frame to a B cell epitope portion comprising one or more B cell epitopes of a non-endogenous CETP. Such non-endogenous, vaccinating B cell epitopes encoded by plasmids of this invention may be derived from another species, another allele, or of non-natural origin (i.e., a synthetic sequence); in such cases the amino acid sequence of the non-endogenous, vaccinating B cell epitope(s) is slightly different from that of the endogenous CETP of the individual to be vaccinated. For example, a vaccinating, non-endogenous B cell epitope that is slightly different from that of a B cell epitope of the endogenous CETP protein is one which has an amino acid sequence which differs from the corresponding B cell epitope of the endogenous CETP at a few, for example, 1, 2, 3, 4, 5, or 6, residues.

Another example of a non-endogenous, vaccinating B cell epitope that is slightly different from a B cell epitope of an endogenous CETP is one that contains one or more conservative changes in amino acid sequence at one or more residues known to be important for a CETP activity and/or for antibody binding (see, for example, Hesler et al., *J. Biol. Chem.*, 263: 5020 - 5023 (1988); Wang et al., *J. Biol. Chem.*, 267: 17487 - 17490 (1992); Wang et al., *J. Biol. Chem.*, 268: 1955 - 1959 (1993)) or at one or more residues known to differ at analogous positions in the amino acid sequence of CETP encoded by other alleles or genes of other species (see, for example, Nagashima et al., *J. Lipid Res.*, 29: 1643 - 1649 (1988); Kotake et al., *J. Lipid Res.*, 37: 599 - 605 (1996); Drayna et al., *Nature*, 327: 632 - 634 (1987)).

An example of a plasmid-based vaccine for humans containing non-endogenous, vaccinating B cell epitopes is the above-described plasmid pCMV-CETP/TT which uses DNA sequences encoding B cell epitopes from rabbit CETP. Another example for use in humans, is a similar plasmid where the encoded B cell epitopes are not derived from a particular species, but are synthetic versions that are slightly different from those encoded by the corresponding human CETP DNA sequences.

While not desiring to be bound by any particular theory, the use of one or more non-endogenous, vaccinating B cell epitopes containing an amino acid sequence which is slightly different from that of a B cell epitope of the endogenous protein (i.e., endogenous CETP) may elicit autoantibodies more effectively than if the endogenous B cell epitopes are employed. Such non-endogenous, vaccinating B cell epitopes (1) elicit production of

antibodies in the vaccinated individual and (2) these elicited antibodies, or a subset thereof, bind endogenous CETP. For example, to test whether a plasmid-based vaccine of this invention elicits autoantibodies to CETP in a human, human CETP transgenic mice (for example, commercially available BIODIGM™- CETP mice, Pharmakon USA, Waverly, PA) 5 are vaccinated with a plasmid construct according to this invention and the production of antibodies recognizing human CETP is quantitated. Quantitation of anti-human CETP antibodies is readily determined by a variety of methods, including Western blotting sera from a vaccinated animal to electrophoresed human CETP; or ELISA where sera from the vaccinated animal is assayed for the ability to bind human CETP or peptide fragment(s) 10 thereof; or isolating CETP from the blood of vaccinated animals and assaying for antibody bound to the CETP.

Bacterial cell cultures (*E. coli*) bearing plasmids pCMV-LUC and pCMV-CETP/TT prepared as described above were deposited April 26, 1996 under the terms of the Budapest Treaty with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD. 15 They were assigned accession numbers 98037 and 98038, respectively.

All documents and publications cited above are incorporated herein by reference.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Thomas, Lawrence J.
- (ii) TITLE OF INVENTION: PLASMID-BASED VACCINE FOR
TREATING ATHEROSCLEROSIS
- (iii) NUMBER OF SEQUENCES: 10
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Banner & Witcoff, Ltd.
 - (B) STREET: 75 State Street, Suite 2300
 - (C) CITY: Boston
 - (D) STATE: Massachusetts
 - (E) COUNTRY: USA
 - (F) ZIP: 02109-1807
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: WordPerfect 6.1
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: (not yet assigned)
 - (B) FILING DATE: 01 May 1997 (01.05.97)
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION: 08/640,713
 - (B) FILING DATE: 01 May 1996 (01.05.96)
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION: 08/802,967
 - (B) FILING DATE: 21 February 1997 (21.02.97)
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Leon R. Yankwich
 - (B) REGISTRATION NUMBER: 30,237
 - (C) REFERENCE/DOCKET NUMBER: TCS 414.1 PCT (05872)

(2) INFORMATION FOR SEQ ID NO: 1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1488 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL:
- (iv) ANTI-SENSE:
- (ix) FEATURE: Structural coding sequence for
mature rabbit CETP
 - (A) NAME:
 - (B) LOCATION:
- (x) PUBLICATION INFORMATION:
 - (A) AUTHORS: Nagashima, Mariko, et al.

(B) TITLE: Cloning and mRNA tissue
distribution of rabbit
cholesteryl ester transfer
protein
(C) JOURNAL: J. Lipid Res.
(D) VOLUME: 29
(E) ISSUE:
(F) PAGES: 1643 - 1649
(G) DATE: 1988
(K) RELEVANT RESIDUES IN SEQ ID NO:1: FROM 1 TO
1488

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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CTCCTCGGCC GGGTCAAGTA CGGGCTGCAC AACCTCCAGA TCAGCCACCT	200
GTCCATCGCC AGCAGCCAGG TGGAGCTGGT GGACGCCAAG ACCATCGACG	250
TCGCCATCCA GAACGTGTCC GTGGTCTTCA AGGGGACCCT GAACTACAGC	300
TACACGAGTG CCTGGGGGTT GGGCATCAAT CAGTCTGTCTG ACTTCGAGAT	350
CGACTCTGCC ATTGACCTCC AGATCAACAC AGAGCTGACC TGCGACGCTG	400
GCAGTGTGCG CACCAATGCC CCCGACTGCT ACCTGGCTTT CCATAAACTG	450
CTCCTGCACC TCCAGGGGGA GCGCGAGCCG GGGTGGCTCA AGCAGCTCTT	500
CACAAACTTC ATCTCCTTCA CCCTGAAGCT GATTCTGAAG CGACAGGTCT	550
GCAATGAGAT CAACACCATC TCCAACATCA TGGCTGACTT TGTCCAGACG	600
AGGGCCGCCA GCATCCTCTC AGATGGAGAC ATCGGGGTGG ACATTTCCGT	650
GACGGGGGCC CCTGTCATCA CAGCCACCTA CCTGGAGTCC CATCACAAGG	700
GTCACTTCAC GCACAAGAAC GTCTCCGAGG CTTCCCCCT CCGCGCCTTC	750
CCGCCCCGTC TTCTGGGGGA CTCCCGCATG CTCTACTTCT GGTTCCTCGA	800
TCAAGTGCTC AACTCCCTGG CCAGGGCCGC CTTCCAGGAG GGCCGTCTCG	850
TGCTCAGCCT GACAGGGGAT GAGTTCAAGA AAGTGCTGGA GACCCAGGGT	900
TTCGACACCA ACCAGGAAAT CTTCCAGGAG CTTTCCAGAG GCCTTCCCAC	950
CGGCCAGGCC CAGGTAGCCG TCCACTGCCT TAAGGTGCCC AAGATCTCCT	1000
GCCAGAACCG GGGTGTCTGT GTGTCTTCTT CCGTCGCCGT GACGTTCCGC	1050
TTCCCCCGCC CAGATGGCCG AGAAGCTGTG GCCTACAGGT TTGAGGAGGA	1100

TATCATCACC ACCGTCCAGG CCTCCTACTC CCAGAAAAAG CTCTTCCTAC 1150
 ACCTCTTGGA TTTCCAGTGC GTGCCGGCCA GCGGAAGGGC AGGCAGCTCA 1200
 GCAAATCTCT CCGTGGCCCT CAGGACTGAG GCTAAGGCTG TTTCCAACCT 1250
 GACTGAGAGC CGCTCCGAGT CCCTGCAGAG CTCTCTCCGC TCCCTGATCG 1300
 CCACGGTGGG CATCCCGGAG GTCATGTCTC GGCTCGAGGT GGC GTTCACA 1350
 GCCCTCATGA ACAGCAAAGG CCTGGACCTC TTCGAAATCA TCAACCCCGA 1400
 GATTATCACT CTCGATGGCT GCCTGCTGCT GCAGATGGAC TTCGGTTTTT 1450
 CCAAGCACCT GCTGGTGGAT TTCCTGCAGA GCCTGAGC 1488

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 496 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL:

(iv) ANTI-SENSE:

(ix) FEATURE:

(A) NAME: Amino acid sequence for mature rabbit CETP protein.

(B) LOCATION:

(x) PUBLICATION INFORMATION:

(A) AUTHORS: Nagashima, Mariko, et al.

(B) TITLE: Cloning and mRNA tissue distribution of rabbit cholesteryl ester transfer protein

(C) JOURNAL: J. Lipid Res.

(D) VOLUME: 29

(E) ISSUE:

(F) PAGES: 1643 - 1649

(G) DATE: 1988

(K) RELEVANT RESIDUES IN SEQ ID NO:2: FROM 1 TO 496

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Cys Pro Lys Gly Ala Ser Tyr Glu Ala Gly Ile Val Cys
 1 5 10

Arg Ile Thr Lys Pro Ala Leu Leu Val Leu Asn Gln Glu
 15 20 25

Thr Ala Lys Val Val Gln Thr Ala Phe Gln Arg Ala Gly
 30 35

Tyr Pro Asp Val Ser Gly Glu Arg Ala Val Met Leu Leu

40		45		50
Gly Arg Val Lys Tyr Gly Leu His Asn Leu Gln Ile Ser	55	60		65
His Leu Ser Ile Ala Ser Ser Gln Val Glu Leu Val Asp	70	75		
Ala Lys Thr Ile Asp Val Ala Ile Gln Asn Val Ser Val	80	85		90
Val Phe Lys Gly Thr Leu Asn Tyr Ser Tyr Thr Ser Ala	95	100		
Trp Gly Leu Gly Ile Asn Gln Ser Val Asp Phe Glu Ile	105	110		115
Asp Ser Ala Ile Asp Leu Gln Ile Asn Thr Glu Leu Thr	120	125		130
Cys Asp Ala Gly Ser Val Arg Thr Asn Ala Pro Asp Cys	135	140		
Tyr Leu Ala Phe His Lys Leu Leu Leu His Leu Gln Gly	145	150		155
Glu Arg Glu Pro Gly Trp Leu Lys Gln Leu Phe Thr Asn	160	165		
Phe Ile Ser Phe Thr Leu Lys Leu Ile Leu Lys Arg Gln	170	175		180
Val Cys Asn Glu Ile Asn Thr Ile Ser Asn Ile Met Ala	185	190		195
Asp Phe Val Gln Thr Arg Ala Ala Ser Ile Leu Ser Asp	200	205		
Gly Asp Ile Gly Val Asp Ile Ser Val Thr Gly Ala Pro	210	215		220
Val Ile Thr Ala Thr Tyr Leu Glu Ser His His Lys Gly	225	230		
His Phe Thr His Lys Asn Val Ser Glu Ala Phe Pro Leu	235	240		245
Arg Ala Phe Pro Pro Gly Leu Leu Gly Asp Ser Arg Met	250	255		260
Leu Tyr Phe Trp Phe Ser Asp Gln Val Leu Asn Ser Leu	265	270		
Ala Arg Ala Ala Phe Gln Glu Gly Arg Leu Val Leu Ser	275	280		285
Leu Thr Gly Asp Glu Phe Lys Lys Val Leu Glu Thr Gln				

34

290

295

[illegible]

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

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SEQUENCE CHARACTERISTICS:
(A) LENGTH:      1428 base pairs
(B) TYPE:        nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY:    linear

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(ii)      MOLECULE TYPE:          cDNA
(iii)     HYPOTHETICAL:
(iv)      ANTI-SENSE:
(ix)      FEATURE:
          (A) NAME:               Structural coding sequence for
                                mature human CETP
          (B) LOCATION:
(x)       PUBLICATION INFORMATION:
          (A) AUTHORS:           Drayna, Dennis, et al.
          (B) TITLE:             Cloning and sequencing of
                                human cholesteryl ester
                                transfer cDNA
          (C) JOURNAL:           Nature
          (D) VOLUME:            327
          (E) ISSUE:
          (F) PAGES:              632 - 634
          (G) DATE:              18-JUN-1987
          (K) RELEVANT RESIDUES IN SEQ ID NO:3:  FROM 1 TO
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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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CTCCTTGGCC	AAGTCAAGTA	TGGGTTCAC	AACATCCAGA	TCAGCCACTT	200
GTCCATCGCC	AGCAGCCAGG	TGGAGCTGGT	GGAAGCCAAG	TCCATTGATG	250
TCTCCATTCA	GAACGTGTCT	GTGGTCTTCA	AGGGGACCCT	GAAGTATGGC	300
TACACCACTG	CCTGGTGGCT	GGGTATTGAT	CAGTCCATTG	ACTTCGAGAT	350
CGACTCTGCC	ATTGACCTCC	AGATCAACAC	ACAGCTGACC	TGTGACTCTG	400
GTAGAGTGCG	GACCGATGCC	CCTGACTGCT	ACCTGTCTTT	CCATAAGCTG	450
CTCCTGCATC	TCCAAGGGGA	GCGAGAGCCT	GGGTGGATCA	AGCAGCTGTT	500
CACAAATTTT	ATCTCCTTCA	CCCTGAAGCT	GGTCCTGAAG	GGACAGATCT	550
GCAAAGAGAT	CAACGTCATC	TCTAACATCA	TGGCCGATTT	TGTCCAGACA	600
AGGGCTGCCA	GCATCCTTTT	AGATGGAGAC	ATTGGGGTGG	ACATTTCCCT	650
GACAGGTGAT	CCCGTCATCA	CAGCCTCCTA	CCTGGAGTCC	CATCACAAGG	700
GTCATTTTCA	CTACAAGAAT	GTCTCAGAGG	ACCTCCCCCT	CCCCACCTTC	750
TCGCCCACAC	TGCTGGGGGA	CTCCCGCATG	CTGTACTTCT	GGTTCTCTGA	800
GCGAGTCTTC	CACTCGCTGG	CCAAGGTAGC	TTTCCAGGAT	GGCCGCCTCA	850
TGCTCAGCCT	GATGGGAGAC	GAGTTCAAGG	CAGTGCTGGA	GACCTGGGGC	900

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TTCAACACCA ACCAGGAAAT CTTCCAAGAG GTTGTGCGCG GCTTCCCCAG      950
CCAGGCCCAA GTCACCGTCC ACTGCCTCAA GATGCCCAAG ATCTCCTGCC      1000
AAAACAAGGG AGTCGTGGTC AATTCTTCAG TGATGGTGAA ATTCCTCTTT      1050
CCACGCCCAG ACCAGCAACA TTCTGTAGCT TACACATTTG AAGAGGATAT      1100
CGTGACTACC GTCCAGGCCT CCTATTCTAA GAAAAAGCTC TTCTTAAGCC      1150
TCTTGGATTT CCAGATTACA CCAAGACTG TTTCCAATT GACTGAGAGC      1200
AGCTCCGAGT CCATCCAGAG CTTCTGCAG TCAATGATCA CCGCTGTGGG      1250
CATCCCTGAG GTCATGTCTC GGCTCGAGGT AGTGTTTACA GCCCTCATGA      1300
ACAGCAAAGG CGTGAGCCTC TTCGACATCA TCAACCCTGA GATTATCACT      1350
CGAGATGGCT TCCTGCTGCT GCAGATGGAC TTTGGCTTCC CTGAGCACCT      1400
GCTGGTGGAT TTCCTCCAGA GCTTGAGC                                1428

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(2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 476 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL:
- (iv) ANTI-SENSE:
- (ix) FEATURE:
 - (A) NAME: Amino acid sequence of mature human CETP
 - (B) LOCATION:
- (x) PUBLICATION INFORMATION:
 - (A) AUTHORS: Drayna, Dennis, et al.
 - (B) TITLE: Cloning and sequencing of human cholesteryl ester transfer cDNA
 - (C) JOURNAL: Nature
 - (D) VOLUME: 327
 - (E) ISSUE:
 - (F) PAGES: 632 - 634
 - (G) DATE: 18-JUN-1987
 - (K) RELEVANT RESIDUES IN SEQ ID NO:4: FROM 1 TO 476

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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Cys Ser Lys Gly Thr Ser His Glu Ala Gly Ile Val Cys
1           5           10
Arg Ile Thr Lys Pro Ala Leu Leu Val Leu Asn His Glu
15           20           25
Thr Ala Lys Val Ile Gln Thr Ala Phe Gln Arg Ala Ser

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37

30

35

Tyr Pro Asp Ile Thr Gly Glu Lys Ala Met Met Leu Leu
 40 45 50
 Gly Gln Val Lys Tyr Gly Leu His Asn Ile Gln Ile Ser
 55 60 65
 His Leu Ser Ile Ala Ser Ser Gln Val Glu Leu Val Glu
 70 75
 Ala Lys Ser Ile Asp Val Ser Ile Gln Asn Val Ser Val
 80 85 90
 Val Phe Lys Gly Thr Leu Lys Tyr Gly Tyr Thr Thr Ala
 95 100
 Trp Trp Leu Gly Ile Asp Gln Ser Ile Asp Phe Glu Ile
 105 110 115
 Asp Ser Ala Ile Asp Leu Gln Ile Asn Thr Gln Leu Thr
 120 125 130
 Cys Asp Ser Gly Arg Val Arg Thr Asp Ala Pro Asp Cys
 135 140
 Tyr Leu Ser Phe His Lys Leu Leu Leu His Leu Gln Gly
 145 150 155
 Glu Arg Glu Pro Gly Trp Ile Lys Gln Leu Phe Thr Asn
 160 165
 Phe Ile Ser Phe Thr Leu Lys Leu Val Leu Lys Gly Gln
 170 175 180
 Ile Cys Lys Glu Ile Asn Val Ile Ser Asn Ile Met Ala
 185 190 195
 Asp Phe Val Gln Thr Arg Ala Ala Ser Ile Leu Ser Asp
 200 205
 Gly Asp Ile Gly Val Asp Ile Ser Leu Thr Gly Asp Pro
 210 215 220
 Val Ile Thr Ala Ser Tyr Leu Glu Ser His His Lys Gly
 225 230
 His Phe Ile Tyr Lys Asn Val Ser Glu Asp Leu Pro Leu
 235 240 245
 Pro Thr Phe Ser Pro Thr Leu Leu Gly Asp Ser Arg Met
 250 255 260
 Leu Tyr Phe Trp Phe Ser Glu Arg Val Phe His Ser Leu
 265 270
 Ala Lys Val Ala Phe Gln Asp Gly Arg Leu Met Leu Ser
 275 280 285

Leu Met Gly Asp Glu Phe Lys Ala Val Leu Glu Thr Trp
 290 295
 Gly Phe Asn Thr Asn Gln Glu Ile Phe Gln Glu Val Val
 300 305 310
 Gly Gly Phe Pro Ser Gln Ala Gln Val Thr Val His Cys
 315 320 325
 Leu Lys Met Pro Lys Ile Ser Cys Gln Asn Lys Gly Val
 330 335
 Val Val Asn Ser Ser Val Met Val Lys Phe Leu Phe Pro
 340 345 350
 Arg Pro Asp Gln Gln His Ser Val Ala Tyr Thr Phe Glu
 355 360
 Glu Asp Ile Val Thr Thr Val Gln Ala Ser Tyr Ser Lys
 365 370 375
 Lys Lys Leu Phe Leu Ser Leu Leu Asp Phe Gln Ile Thr
 380 385 390
 Pro Lys Thr Val Ser Asn Leu Thr Glu Ser Ser Ser Glu
 395 400
 Ser Ile Gln Ser Phe Leu Gln Ser Met Ile Thr Ala Val
 405 410 415
 Gly Ile Pro Glu Val Met Ser Arg Leu Glu Val Val Phe
 420 425
 Thr Ala Leu Met Asn Ser Lys Gly Val Ser Leu Phe Asp
 430 435 440
 Ile Ile Asn Pro Glu Ile Ile Thr Arg Asp Gly Phe Leu
 445 450 455
 Leu Leu Gln Met Asp Phe Gly Phe Pro Glu His Leu Leu
 460 465
 Val Asp Phe Leu Gln Ser Leu Ser
 470 475

(2) INFORMATION FOR SEQ ID NO: 5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 169 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE:
- (iii) HYPOTHETICAL:
- (iv) ANTI-SENSE:
- (ix) FEATURE:

- (A) NAME:
(B) LOCATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCGGCCGCCA TGCAGTACAT CAAGGCCAAC TCCAAGTTCA TCGGCATCAC 50
GGAGCGCTTC CCCC GCCCAG ATGGCCGAGA AGCTGTGGCC TACAGGTTTG 100
AGGAGGATAT CTTCGGTTTT CCCAAGCACC TGCTGGTGGA TTTCCTGCAG 150
AGCCTGAGCT AGCGGCCGC 169

(2) INFORMATION FOR SEQ ID NO: 6:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 169 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: DNA
 (iii) HYPOTHETICAL:
 (iv) ANTI-SENSE:
 (ix) FEATURE:
 (A) NAME: Complementary strand to SEQ ID NO:5
 (B) LOCATION: 1 to 169

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCGGCCGCTA GCTCAGGCTC TGCAGGAAAT CCACCAGCAG GTGCTTGGA 50
AAACCGAAGA TATCCTCCTC AAACCTGTAG GCCACAGCTT CTCGGCCATC 100
TGGGCGGGGG AAGCGCTCCG TGATGCCGAT GAACTTGAG TTGGCCTTGA 150
TGTACTGCAT CGCGGCCGC 169

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 50 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: peptide
 (iii) HYPOTHETICAL:
 (iv) ANTI-SENSE:
 (ix) FEATURE: amino acid sequence of peptide encoded
 by bases 10 to 159 of SEQ ID NO:5
 (A) NAME:
 (B) LOCATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Met Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile
1 5 10
Thr Glu Arg Phe Pro Arg Pro Asp Gly Arg Glu Ala Val
15 20 25

Ala Tyr Arg Phe Glu Glu Asp Ile Phe Gly Phe Pro Lys
30 35

His Leu Leu Val Asp Phe Leu Gln Ser Leu Ser
40 45 50

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1608 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: Single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE:
- (iii) HYPOTHETICAL:
- (iv) ANTI-SENSE:
- (ix) FEATURE:
 - (A) NAME: translational stop codon
 - (B) LOCATION: 1606 - 1608
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGCGCTGATG ATGTTGTTGA TTCTTCTAAA TCTTTTGTGA TGGAAACTT	50
TTCTTCGTAC CACGGGACTA AACCTGGTTA TGTAAGATTCC ATTCAAAAAG	100
GTATACAAAA GCCAAAATCT GGTACACAAG GAAATTATGA CGATGATTGG	150
AAAGGGTTTT ATAGTACCGA CAATAAATAC GACGCTGCGG GATACTCTGT	200
AGATAATGAA AACCCGCTCT CTGGAAAAGC TGGAGGCGTG GTCAAAGTGA	250
CGTATCCAGG ACTGACGAAG GTTCTCGCAC TAAAAGTGGA TAATGCCGAA	300
ACTATTAAGA AAGAGTTAGG TTTAAGTCTC ACTGAACCGT TGATGGAGCA	350
AGTCGGAACG GAAGAGTTTA TCAAAAGGTT CGGTGATGGT GCTTCGCGTG	400
TAGTGCTCAG CCTTCCCTTC GCTGAGGGGA GTTCTAGCGT TGAATATATT	450
AATAACTGGG AACAGGCGAA AGCGTTAAGC GTAGAACTTG AGATTAATTT	500
TGAAACCCGT GGAAAACGTG GCCAAGATGC GATGTATGAG TATATGGCTC	550
AAGCCTGTGC AGGAAATCGT GTCAGGCGAT CAGTAGGTAG CTCATTGTCA	600

TGCATAAATC TTGATTGGGA TGTCATAAGG GATAAAACTA AGACAAAGAT	650
AGAGTCTTTG AAAGAGCATG GCCCTATCAA AAATAAAATG AGCGAAAGTC	700
CCAATAAAAC AGTATCTGAG GAAAAAGCTA AACAATACCT AGAAGAATTT	750
CATCAAACGG CATTAGAGCA TCCTGAATTG TCAGAACTTA AAACCGTTAC	800
TGGGACCAAT CCTGTATTCTG CTGGGGCTAA CTATGCGGCG TGGGCAGTAA	850
ACGTTGCGCA AGTTATCGAT AGCGAAACAG CTGATAATTT GGAAAAGACA	900
ACTGCTGCTC TTTCGATACT TCCTGGTATC GGTAGCGTAA TGGGCATTGC	950
AGACGGTGCC GTTCACCACA ATACAGAAGA GATAGTGGCA CAATCAATAG	1000
CTTTATCGTC TTTAATGGTT GCTCAAGCTA TTCCATTGGT AGGAGAGCTA	1050
GTTGATATTG GTTTCGCTGC ATATAATTTT GTAGAGAGTA TTATCAATTT	1100
ATTTCAAGTA GTTCATAATT CGTATAATCG TCCCGCGTAT TCTCCGGGGC	1150
ATAAAACGCA ACCATTCTT CATGACGGGT ATGCTGTCAG TTGGAACACT	1200
GTTGAAGATT CGATAATCCG AACTGGTTTT CAAGGGGAGA GTGGGCACGA	1250
CATAAAAATT ACTGCTGAAA ATACCCCGCT TCCAATCGCG GGTGTCCTAC	1300
TACCGACTAT TCCTGGAAAG CTGGACGTTA ATAAGTCCAA GACTCATATT	1350
TCCGTAAATG GTCGGAAAAT AAGGATGCGT TGCAGAGCTA TAGACGGTGA	1400
TGTAACTTTT TGTCGCCCTA AATCTCCTGT TTATGTTGGT AATGGTGTGC	1450
ATGCGAATCT TCACGTGGCA TTTCACAGAA GCAGCTCGGA GAAAATTCAT	1500
TCTAATGAAA TTTCGTCGGA TTCCATAGGC GTTCTTGGGT ACCAGAAAAC	1550

AGTAGATCAC ACCAAGGTTA ATTCTAAGCT ATCGCTATTT TTTGAAATCA 1600

AAAGCTGA 1608

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 535 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (iii) HYPOTHETICAL:
- (iv) ANTI-SENSE:
- (ix) FEATURE:
 - (A) NAME:
 - (B) LOCATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Gly	Ala	Asp	Asp	Val	Val	Asp	Ser	Ser	Lys	Ser	Phe	Val	Met
1				5					10				
Glu	Asn	Phe	Ser	Ser	Tyr	His	Gly	Thr	Lys	Pro	Gly	Tyr	Val
15					20				25				
Asp	Ser	Ile	Gln	Lys	Gly	Ile	Gln	Lys	Pro	Lys	Ser	Gly	Thr
	30				35						40		
Gln	Gly	Asn	Tyr	Asp	Asp	Asp	Trp	Lys	Gly	Phe	Tyr	Ser	Thr
		45					50					55	
Asp	Asn	Lys	Tyr	Asp	Ala	Ala	Gly	Tyr	Ser	Val	Asp	Asn	Glu
		60					65					70	
Asn	Pro	Leu	Ser	Gly	Lys	Ala	Gly	Gly	Val	Val	Lys	Val	Thr
			75						80				
Tyr	Pro	Gly	Leu	Thr	Lys	Val	Leu	Ala	Leu	Lys	Val	Asp	Asn
85					90					95			
Ala	Glu	Thr	Ile	Lys	Lys	Glu	Leu	Gly	Leu	Ser	Leu	Thr	Glu
	100					105					110		
Pro	Leu	Met	Glu	Gln	Val	Gly	Thr	Glu	Glu	Phe	Ile	Lys	Arg
		115					120					125	
Phe	Gly	Asp	Gly	Ala	Ser	Arg	Val	Val	Leu	Ser	Leu	Pro	Phe
		130						135					140
Ala	Glu	Gly	Ser	Ser	Ser	Val	Glu	Tyr	Ile	Asn	Asn	Trp	Glu
			145					150					
Gln	Ala	Lys	Ala	Leu	Ser	Val	Glu	Leu	Glu	Ile	Asn	Phe	Glu
155					160					165			
Thr	Arg	Gly	Lys	Arg	Gly	Gln	Asp	Ala	Met	Tyr	Glu	Tyr	Met

170		175		180
Ala Gln Ala Cys Ala Gly Asn Arg Val Arg Arg Ser Val Gly	185		190	195
Ser Ser Leu Ser Cys Ile Asn Leu Asp Trp Asp Val Ile Arg	200		205	210
Asp Lys Thr Lys Thr Lys Ile Glu Ser Leu Lys Glu His Gly		215		220
Pro Ile Lys Asn Lys Met Ser Glu Ser Pro Asn Lys Thr Val	225	230		235
Ser Glu Glu Lys Ala Lys Gln Tyr Leu Glu Glu Phe His Gln	240		245	250
Thr Ala Leu Glu His Pro Glu Leu Ser Glu Leu Lys Thr Val	255		260	265
Thr Gly Thr Asn Pro Val Phe Ala Gly Ala Asn Tyr Ala Ala		270	275	280
Trp Ala Val Asn Val Ala Gln Val Ile Asp Ser Glu Thr Ala		285	290	
Asp Asn Leu Glu Lys Thr Thr Ala Ala Leu Ser Ile Leu Pro	295	300		305
Gly Ile Gly Ser Val Met Gly Ile Ala Asp Gly Ala Val His	310		315	320
His Asn Thr Glu Glu Ile Val Ala Gln Ser Ile Ala Leu Ser	325		330	335
Ser Leu Met Val Ala Gln Ala Ile Pro Leu Val Gly Glu Leu	340		345	350
Val Asp Ile Gly Phe Ala Ala Tyr Asn Phe Val Glu Ser Ile		355	360	
Ile Asn Leu Phe Gln Val Val His Asn Ser Tyr Asn Arg Pro	365	370		375
Ala Tyr Ser Pro Gly His Lys Thr Gln Pro Phe Leu His Asp	380	385		390
Gly Tyr Ala Val Ser Trp Asn Thr Val Glu Asp Ser Ile Ile	395		400	405
Arg Thr Gly Phe Gln Gly Glu Ser Gly His Asp Ile Lys Ile	410		415	420
Thr Ala Glu Asn Thr Pro Leu Pro Ile Ala Gly Val Leu Leu		425	430	
Pro Thr Ile Pro Gly Lys Leu Asp Val Asn Lys Ser Lys Thr	435	440		445

His Ile Ser Val Asn Gly Arg Lys Ile Arg Met Arg Cys Arg
 450 455 460

Ala Ile Asp Gly Asp Val Thr Phe Cys Arg Pro Lys Ser Pro
 465 470 475

Val Tyr Val Gly Asn Gly Val His Ala Asn Leu His Val Ala
 480 485 490

Phe His Arg Ser Ser Ser Glu Lys Ile His Ser Asn Glu Ile
 495 500

Ser Ser Asp Ser Ile Gly Val Leu Gly Tyr Gln Lys Thr Val
 505 510 515

Asp His Thr Lys Val Asn Ser Lys Leu Ser Leu Phe Phe Glu
 520 525 530

Ile Lys Ser
 535

(2) INFORMATION FOR SEQ ID NO:10

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (iii) HYPOTHETICAL:
- (iv) ANTI-SENSE:
- (ix) FEATURE:
 - (A) NAME:
 - (B) LOCATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg Val Pro
 1 5 10

Lys Val Ser Ala Ser His Leu Glu
 15 20

CLAIMS:

1. A DNA plasmid-based vaccine comprising a nucleotide sequence coding for an immunogenic polypeptide, which nucleotide sequence includes at least one segment coding for a B cell epitope of CETP linked in-frame with at least one segment coding for a broad range helper T cell epitope, which nucleotide sequence is operably linked to a promoter sequence suitable for directing the transcription of the nucleotide sequence in a mammalian cell.
2. The DNA plasmid-based vaccine according to claim 1 wherein said B cell epitope comprises a portion of human CETP consisting of 5-8 consecutive amino acids of SEQ ID NO:4.
3. The DNA plasmid-based vaccine according to claim 1 wherein said B cell epitope comprises a carboxyl terminal region of CETP, involved in neutral lipid binding or neutral lipid transfer activity.
4. The DNA plasmid-based vaccine according to claim 1 wherein the helper T cell epitope comprises a helper T cell epitope derived from an antigenic peptide selected from the group consisting of tetanus toxoid, diphtheria toxin, pertussis vaccine, Bacille Calmette-Guerin (BCG), polio vaccine, measles vaccine, mumps vaccine, rubella vaccine, purified protein derivative of tuberculin, keyhole limpet hemocyanin, and combinations thereof.
5. The DNA plasmid-based vaccine according to claim 1 wherein the immunogenic polypeptide includes two B cell epitopes of CETP.
6. The DNA plasmid-based vaccine according to claim 5 which includes a DNA segment coding for amino acids 463 through 475 of SEQ ID NO: 4 and a DNA segment coding for amino acids 349 through 367 of SEQ ID NO: 4.
7. The DNA plasmid-based vaccine according to claim 5 which includes a DNA segment coding for amino acids 461 through 476 of SEQ ID NO: 4 and a DNA segment coding for amino acids 349 through 367 of SEQ ID NO: 4.

8. The DNA plasmid-based vaccine according to claim 1 which includes a DNA segment coding for amino acids 2 through 15 of SEQ ID NO: 7.

9. The DNA plasmid-based vaccine according to claim 1 comprising the amino acid sequence of SEQ ID NO:7.

10. A DNA plasmid-based vaccine according to claim 1, wherein the promoter is the cytomegalovirus immediate early promoter/enhancer.

11. A DNA plasmid-based vaccine comprising a nucleotide sequence comprising:
(a) the immediate early promoter/enhancer region of cytomegalovirus (CMV), operably linked to (b) a structural DNA segment encoding an immunogenic polypeptide and comprising:

- (i) a DNA segment encoding amino acids 2 through 15 of SEQ ID NO: 7,
 - (ii) a DNA segment encoding amino acids 463 through 475 of SEQ ID NO: 4, and
 - (iii) a DNA segment encoding amino acids 349 through 367 of SEQ ID NO: 4,
- which DNA segments (i), (ii) and (iii) are linked in-frame.

12. A DNA plasmid-based vaccine comprising a nucleotide sequence comprising:
(a) the immediate early promoter/enhancer region of cytomegalovirus (CMV), operably linked to (b) a structural DNA segment encoding an immunogenic polypeptide and comprising:

- (i) a DNA segment encoding amino acids 2 through 15 of SEQ ID NO: 7,
 - (ii) a DNA segment encoding amino acids 461 through 476 of SEQ ID NO: 4, and
 - (iii) a DNA segment encoding amino acids 349 through 367 of SEQ ID NO: 4,
- which DNA segments (i), (ii) and (iii) are linked in-frame.

13. A DNA plasmid-based vaccine comprising a DNA segment wherein the DNA segment comprises a nucleotide sequence coding for a broad range T cell epitope, the nucleotide sequence of nucleotides 54 through 111 of SEQ ID NO:5, and the nucleotide sequence of 112 through 159 of SEQ ID NO:5

14. The DNA plasmid-based vaccine according to claim 13 wherein the DNA

segment comprises the nucleotide sequence of SEQ ID NO:5.

15. A DNA plasmid-based vaccine comprising a DNA segment wherein the DNA segment comprises a nucleotide sequence encoding a broad range T cell epitope, the nucleotide sequence of nucleotides 1045 through 1101 of SEQ ID NO:3, and nucleotides 1387 through 1425 of SEQ ID NO:3.

16. A DNA plasmid-based vaccine comprising a DNA segment wherein the DNA segment comprises a nucleotide sequence coding for a broad range T cell epitope, the nucleotide sequence of nucleotides 1045 through 1101 of SEQ ID NO:3, and nucleotides 1381 through 1428 of SEQ ID NO:3.

17. A method of elevating the ratio of circulating HDL to circulating LDL, VLDL, or total cholesterol in a human or other animal comprising administering to the human or animal a DNA vaccine comprising a nucleotide sequence coding for an immunogenic polypeptide, which nucleotide sequence includes at least one segment coding for a B cell epitope of CETP linked in-frame with at least one segment coding for a broad range helper T cell epitope, which nucleotide sequence is operably linked to a promoter sequence suitable for directing the transcription of the nucleotide sequence in a mammalian cell.

18. The method according to claim 17 wherein said B cell epitope comprises a carboxyl terminal region of CETP involved in neutral lipid binding or neutral lipid transfer activity.

19. The method according to claim 17 wherein the broad range helper T cell epitope is selected from the group consisting of T cell epitopes of tetanus toxoid, diphtheria toxin, pertussis vaccine, Bacille Calmette-Guerin (BCG), polio vaccine, measles vaccine, mumps vaccine, rubella vaccine, purified protein derivative of tuberculin, and keyhole limpet hemocyanin.

20. The method according to claim 17 wherein the immunogenic polypeptide comprises a B cell epitope from the C-terminal 26 amino acids of human CETP and a T cell epitope from tetanus toxoid.

21. The method according to claim 20 wherein the immunogenic peptide comprises two B cell epitopes of human CETP.

22. A method of decreasing the level of endogenous CETP activity in a human or other animal comprising administering to the human or animal a DNA vaccine comprising a nucleotide sequence coding for an immunogenic polypeptide, which nucleotide sequence includes at least one segment coding for a B cell epitope of CETP linked in-frame with at least one segment coding for a broad range helper T cell epitope, which nucleotide sequence is operably linked to a promoter sequence suitable for directing the transcription of the nucleotide sequence in a mammalian cell.

23. The method according to claim 22 wherein said B cell epitope comprises a carboxyl terminal region of CETP involved in neutral lipid binding or neutral lipid transfer activity.

24. The method according to claim 22 wherein the broad range helper T cell epitope is selected from the group consisting of T cell epitopes of tetanus toxoid, diphtheria toxin, pertussis vaccine, Bacille Calmette-Guerin (BCG), polio vaccine, measles vaccine, mumps vaccine, rubella vaccine, purified protein derivative of tuberculin, and keyhole limpet hemocyanin.

25. The method according to claim 22 wherein the immunogenic polypeptide comprises a B cell epitope from the C-terminal 26 amino acids of human CETP and a T cell epitope from tetanus toxoid.

26. The method according to claim 25 wherein the immunogenic peptide comprises two B cell epitopes of human CETP.

27. A method for eliciting production of anti-CETP antibodies in a human or animal comprising administering a DNA vaccine comprising a nucleotide sequence coding for an immunogenic polypeptide, which nucleotide sequence includes at least one segment coding for a B cell epitope of CETP linked in-frame with at least one segment coding for a broad range helper T cell epitope, which nucleotide sequence is operably linked to a promoter

sequence suitable for directing the transcription of the nucleotide sequence in a mammalian cell.

28. A method of increasing the level of circulating HDL in a human or animal comprising administering to the human or animal a DNA vaccine comprising a nucleotide sequence coding for an immunogenic polypeptide, which nucleotide sequence includes at least one segment coding for a B cell epitope of CETP linked in-frame with at least one segment coding for a broad range helper T cell epitope, which nucleotide sequence is operably linked to a promoter sequence suitable for directing the transcription of the nucleotide sequence in a mammalian cell.

29. The method according to claim 28, wherein the helper T cell epitope comprises a helper T cell epitope derived from an antigenic peptide selected from the group consisting of tetanus toxoid, diphtheria toxin, pertussis vaccine, Bacille Calmette-Guerin (BCG), polio vaccine, measles vaccine, mumps vaccine, rubella vaccine, purified protein derivative of tuberculin, keyhole limpet hemocyanin, and combinations thereof.

30. The method according to claim 28, wherein the B cell epitope portion comprises a carboxyl terminal region of human CETP.

31. A method for therapeutically or prophylactically treating cardiovascular disease in a human or other animal in need of treatment thereof comprising administering to said human or other animal a DNA plasmid-based vaccine comprising a DNA segment comprising the nucleotide sequence coding for an immunogenic polypeptide, which nucleotide sequence includes at least one segment coding for a B cell epitope of CETP linked in-frame with at least one segment coding for a broad range helper T cell epitope, which nucleotide sequence is operably linked to a promoter sequence suitable for directing the transcription of the nucleotide sequence in a mammalian cell.

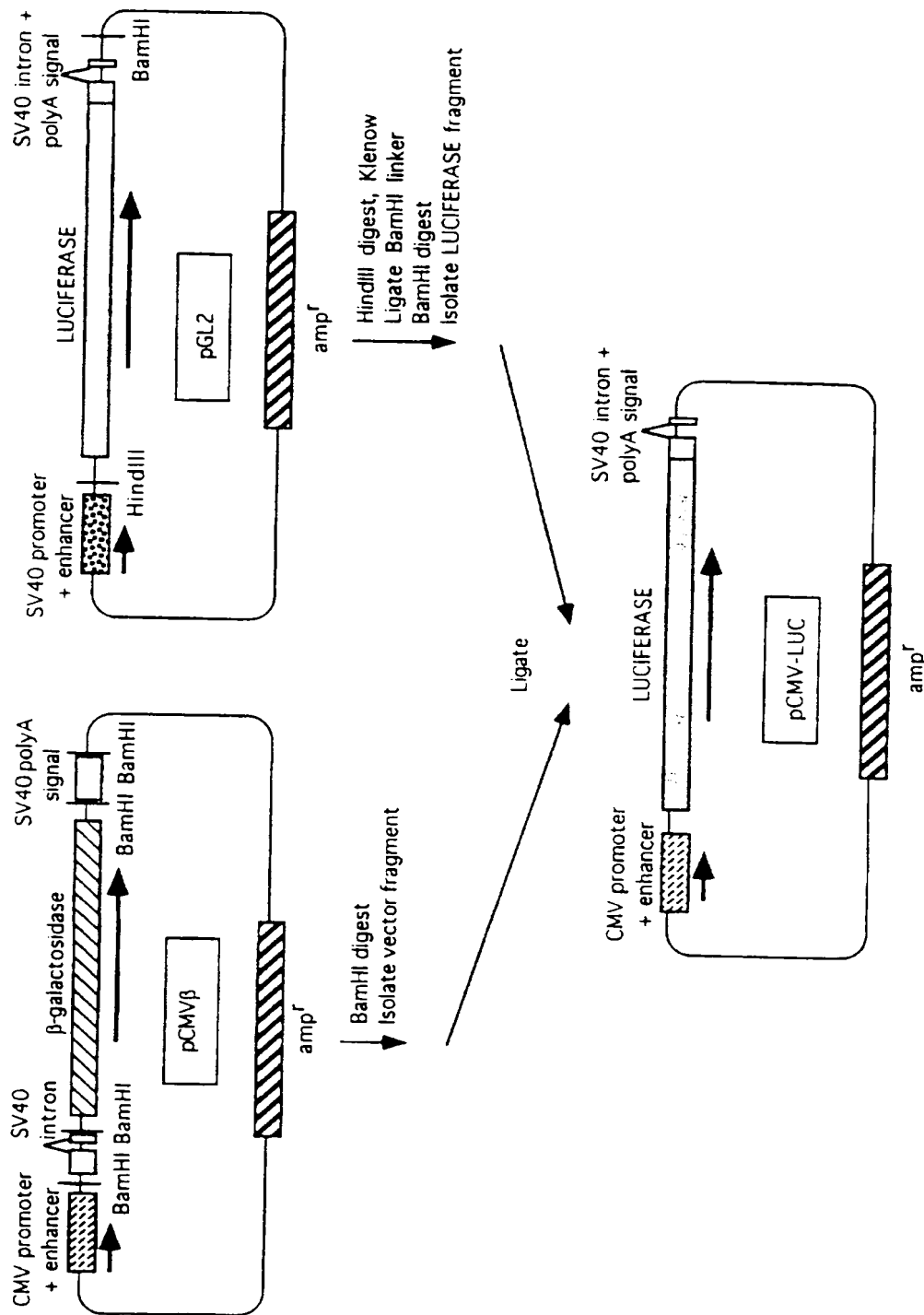
32. The method according to claim 31, wherein the nucleotide sequence coding for an immunogenic polypeptide comprises a DNA sequence of nucleotides 55 through 111 of SEQ ID NO:5 and a DNA sequence of nucleotides 112 through 159 of SEQ ID NO:5.

33. The method according to claim 31, wherein the DNA segment comprises the nucleotide sequence of SEQ ID NO:5.

34. The method according to claim 31, wherein the DNA nucleotide sequence coding for an immunogenic polypeptide comprises the DNA sequence of nucleotides 1045 through 1101 of SEQ ID NO:3 and the DNA sequence of nucleotides 1387 through 1425 of SEQ ID NO:3.

35. The method according to claim 31, wherein the DNA nucleotide sequence coding for an immunogenic polypeptide comprises the DNA sequence of nucleotides 1045 through 1101 of SEQ ID NO:3 and the DNA sequence of nucleotides 1381 through 1428 of SEQ ID NO:3.

FIGURE 1



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FIGURE 2

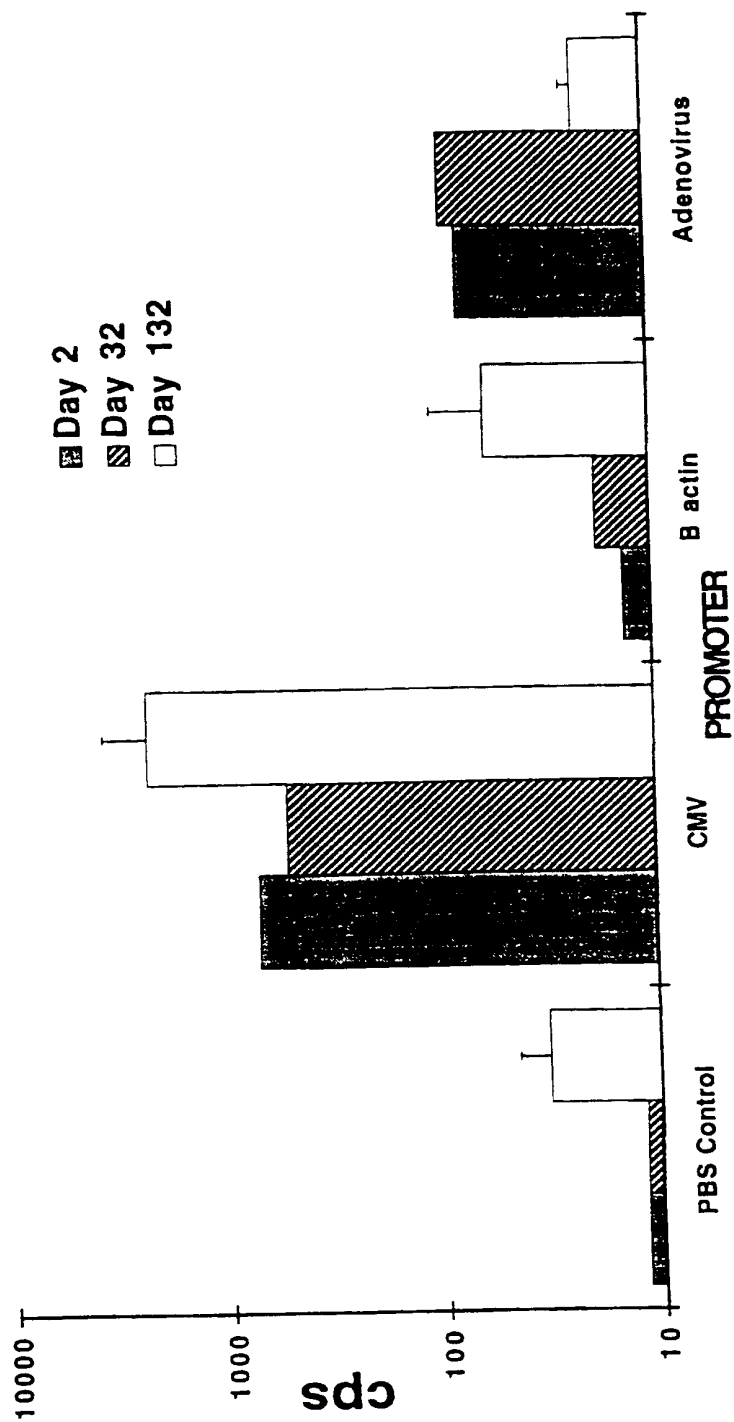
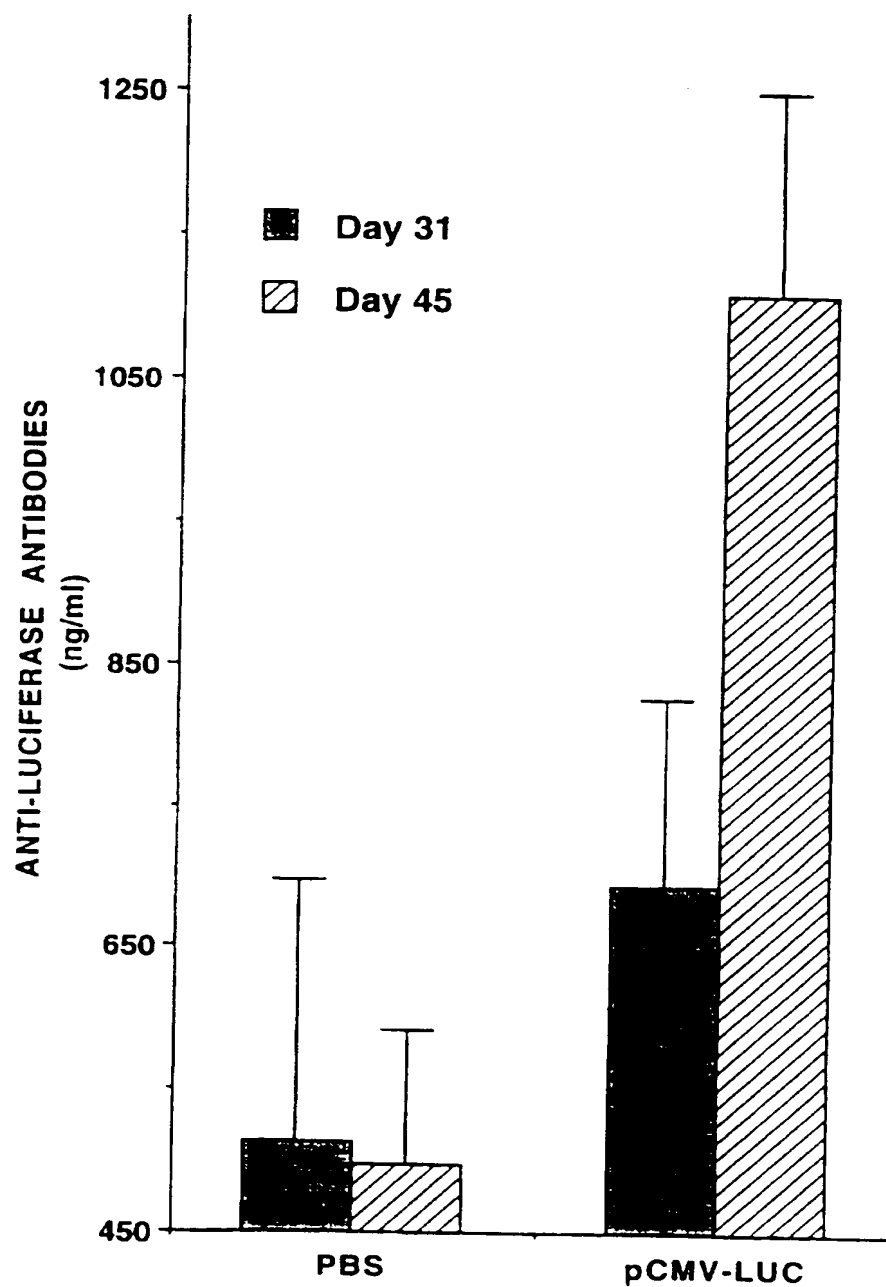
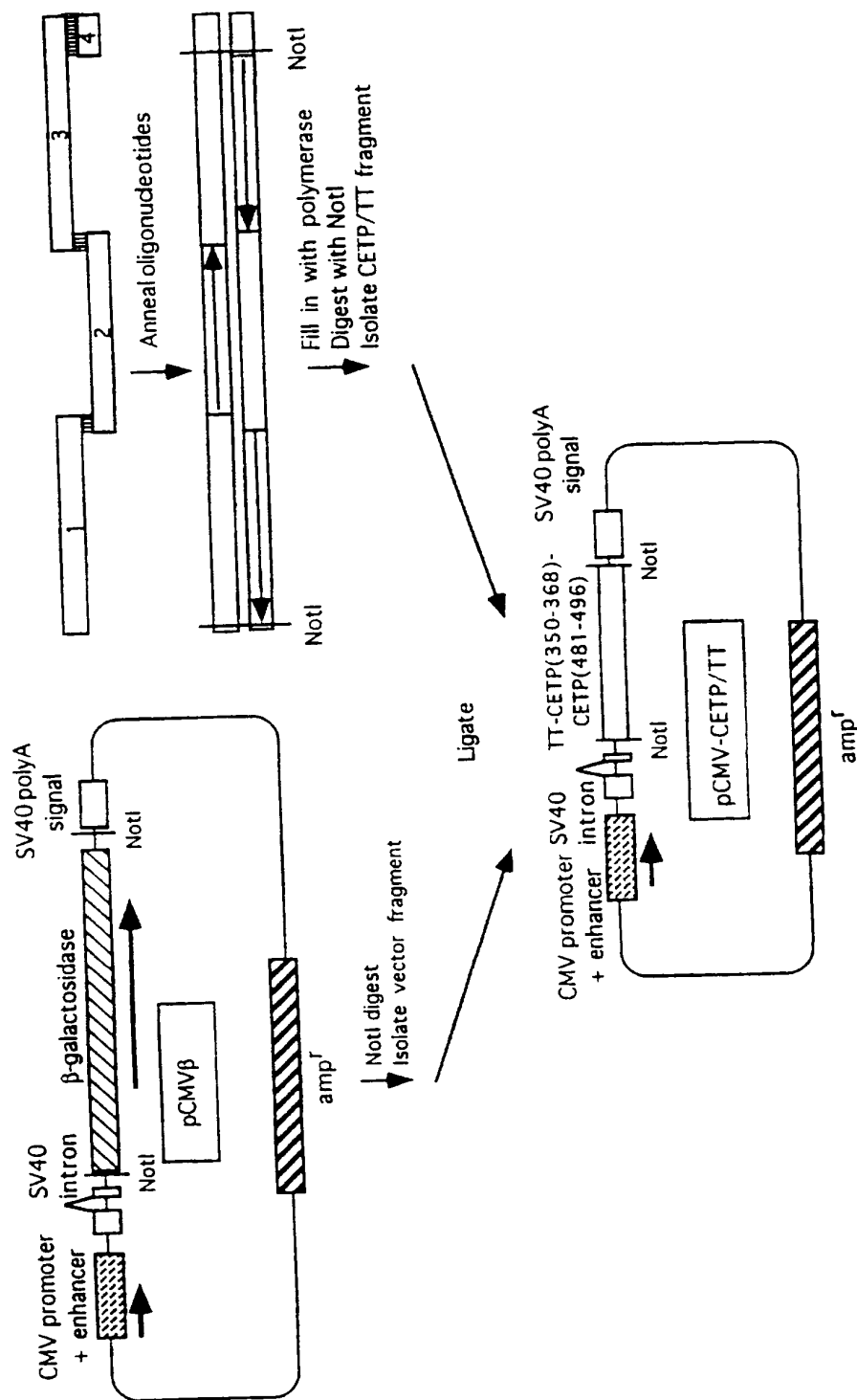


FIGURE 3



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FIGURE 4



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FIGURE 5

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GGGGCCGCC ATG CAG TAC ATC AAG GCC AAC TCC AAG TTC ATC GGC ATC ACG GAG
CGCCGGCGC TAC GTC ATG TAG TTC CGG TTG AGG TTC AAG TAG CCG TAG TGC CTC
NotI      M  Q  Y  I  K  A  N  S  K  F  I  G  I  T  E      >|
          |<
TETANUS TOXOID
CGC TTC CCC CGC CCA GAT GGC CGA GAA GCT GTG GCC TAC AGG TTT GAG GAG GAT ATC
GCG AAG GGG GCG GGT CTA CCG GCT CTT CGA CAC CGG ATG TCC AAA CTC CTC CTA TAG
R  F  P  R  P  D  G  R  E  A  V  A  Y  R  F  E  E  D  I      >|
          |<
CETP (350-368)
TTC GGT TTT CCC AAG CAC CTG CTG GTG GAT TTC CTG CAG AGC CTG AGC TAG CGGCCGC
AAG CCA AAA GGG TTC GTG GAC GAC CAC CTA AAG GAC GTC TCG GAC TCG ATC GCCGGCG
F  G  F  P  K  H  L  L  V  D  F  L  Q  S  L  S  *
          |<
CETP (481-496)
          >| STOP NotI

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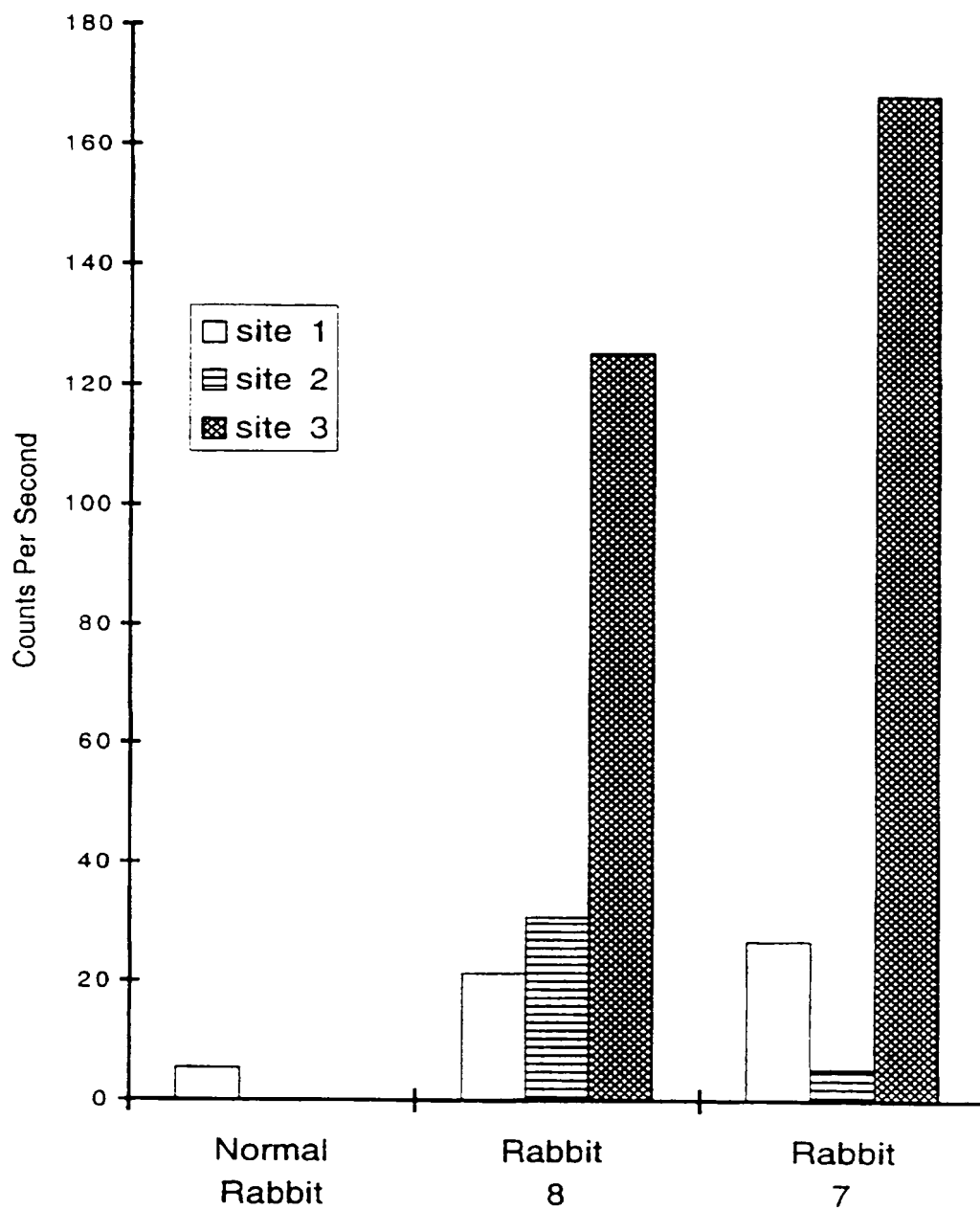
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FIGURE 6

	RABBIT #													
	1	2	3	4	5	6	7	8	10	11	12	13	14	
PRE 1	(-11)	(-11)	(-11)	(-11)	(-11)	(-11)	(-11)	(-11)	X	X	X	X	X	
PRE 2	(-6)	(-6)	(-6)	(-6)	(-6)	(-6)	(-6)	(-6)	X	X	X	X	X	
PRE 3	0	0	0	0	0	0	0	0	X	X	X	X	X	
CETP Vaccine	0	0	0	0	0	0	0	0	X	X	X	X	X	
BLEED 1	14	14	14	14	14	14	14	X	X	X	X	X	X	
BLEED 2	28	28	28	28	28	28	28	X	X	X	X	X	X	
CETP Vaccine	28	28	28	28	28	28	28	X	X	X	X	X	X	
BLEED 3	44	44	44	44	44	44	X	X	X	X	X	X	X	
BLEED 4	57	57	57	57	57	57	X	X	X	X	X	X	X	
Tetanus	66	66	66	66	66	66	X	X	X	X	X	X	X	
Tetanus	91	91	91	91	91	91	X	X	X	X	X	X	X	
BLEED 5	99	99	99	99	99	99	X	X	99	99	99	99	99	
0.25% Chol.	99	99	99	99	99	99	X	X	99	99	99	99	99	
0.5% Chol.	112	112	112	112	112	112	X	X	112	112	112	112	112	
BLEED 6	128	128	128	128	128	128	X	X	128	128	128	128	128	
Tetanus	128	128	128	128	128	128	X	X	X	X	X	X	X	
BLEED 7	147	147	147	147	147	147	X	X	147	147	147	147	147	
0.25% Chol.	X	154	154	X	154	154	X	X	154	X	154	154	X	
BLEED 8	X	163	163	X	163	163	X	X	163	X	163	163	X	
BLEED 9	X	177	177	X	177	177	X	X	177	X	177	177	X	
BLEED 10	X	198	198	X	198	198	X	X	198	X	198	198	X	
BLEED 11	X	212	212	X	212	212	X	X	212	X	212	212	X	
BLEED 12	X	220	220	X	220	220	X	X	220	X	X	220	X	
Termination	148	220	220	148	220	220	30	2	220	148	219	220	147	

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FIGURE 7



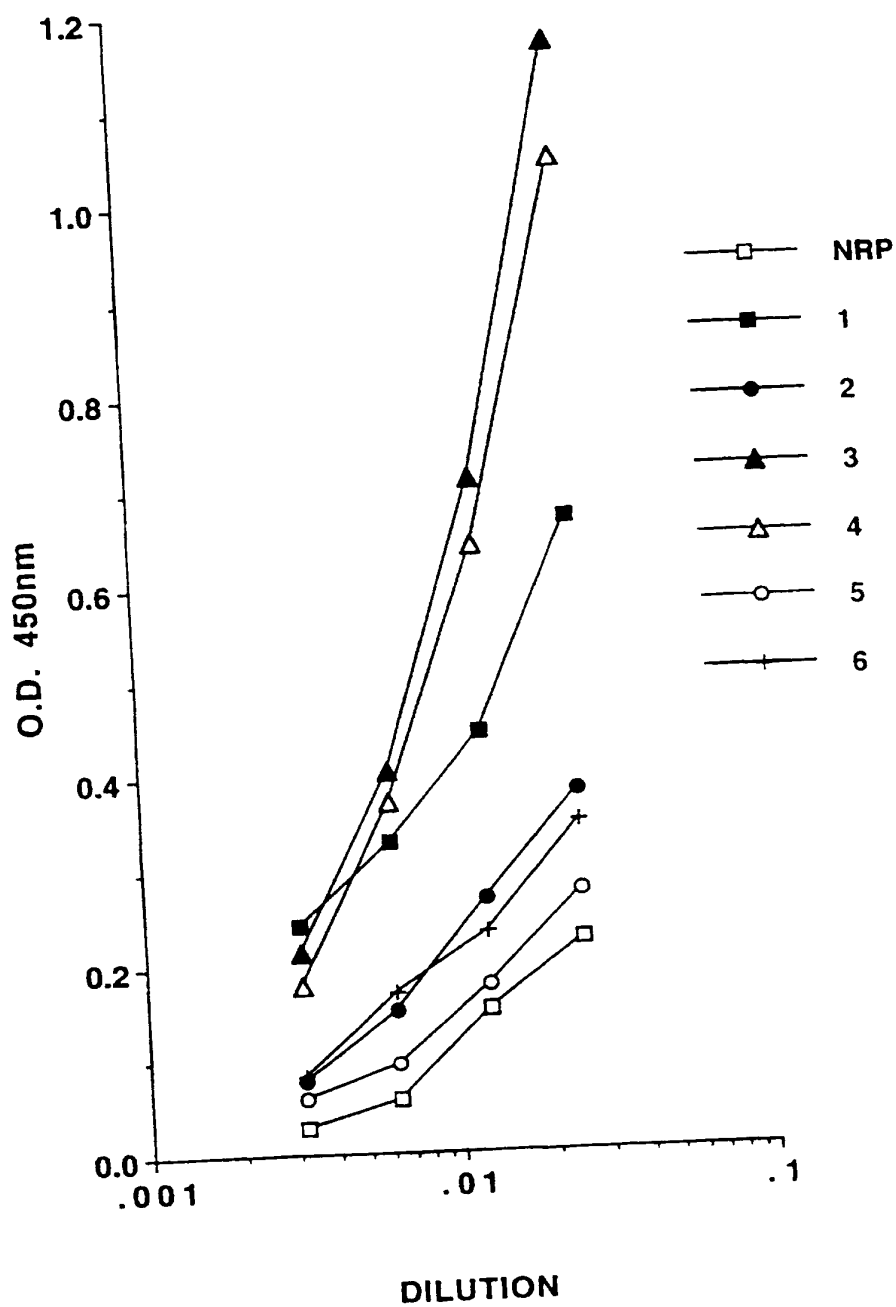
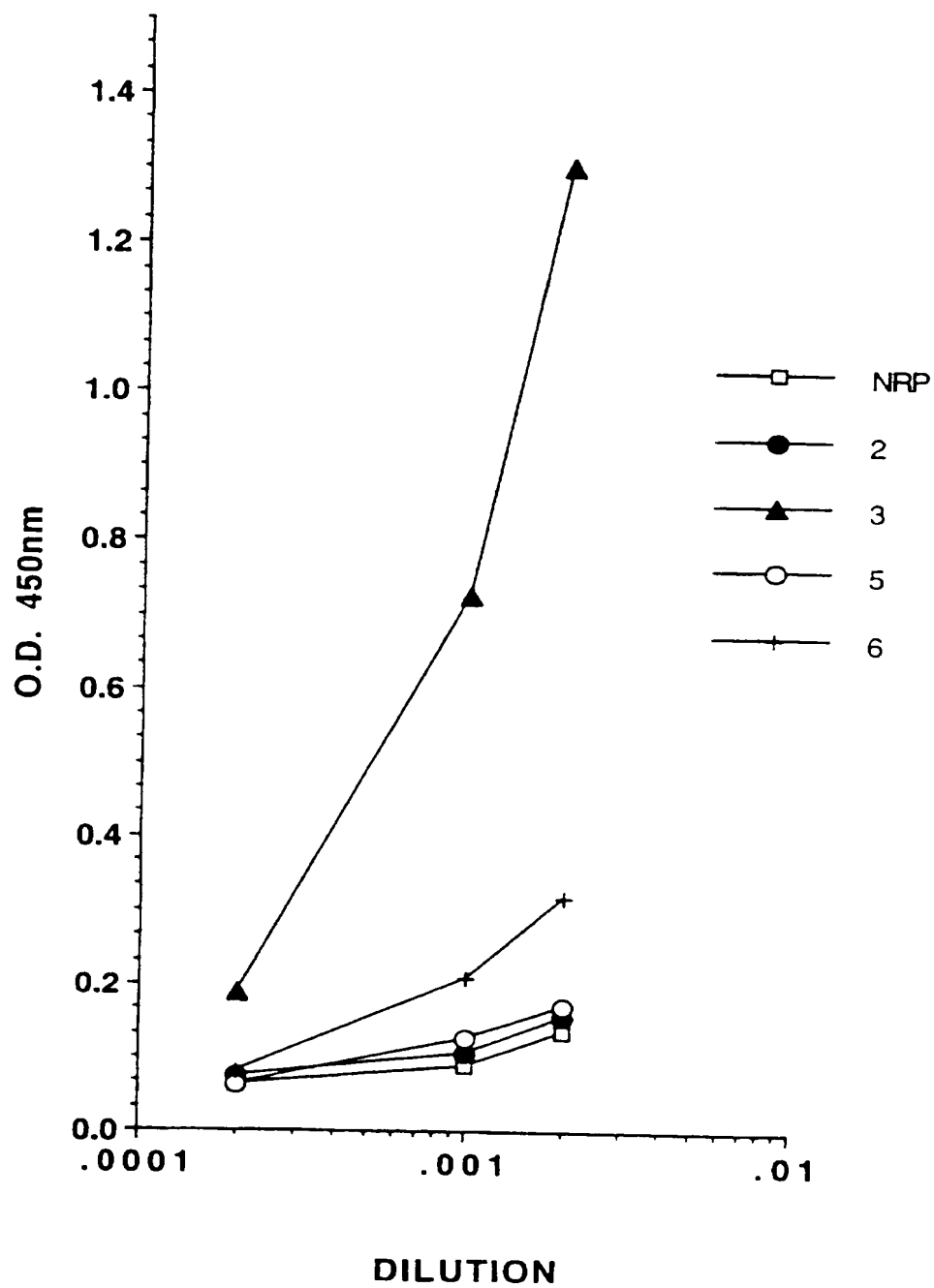
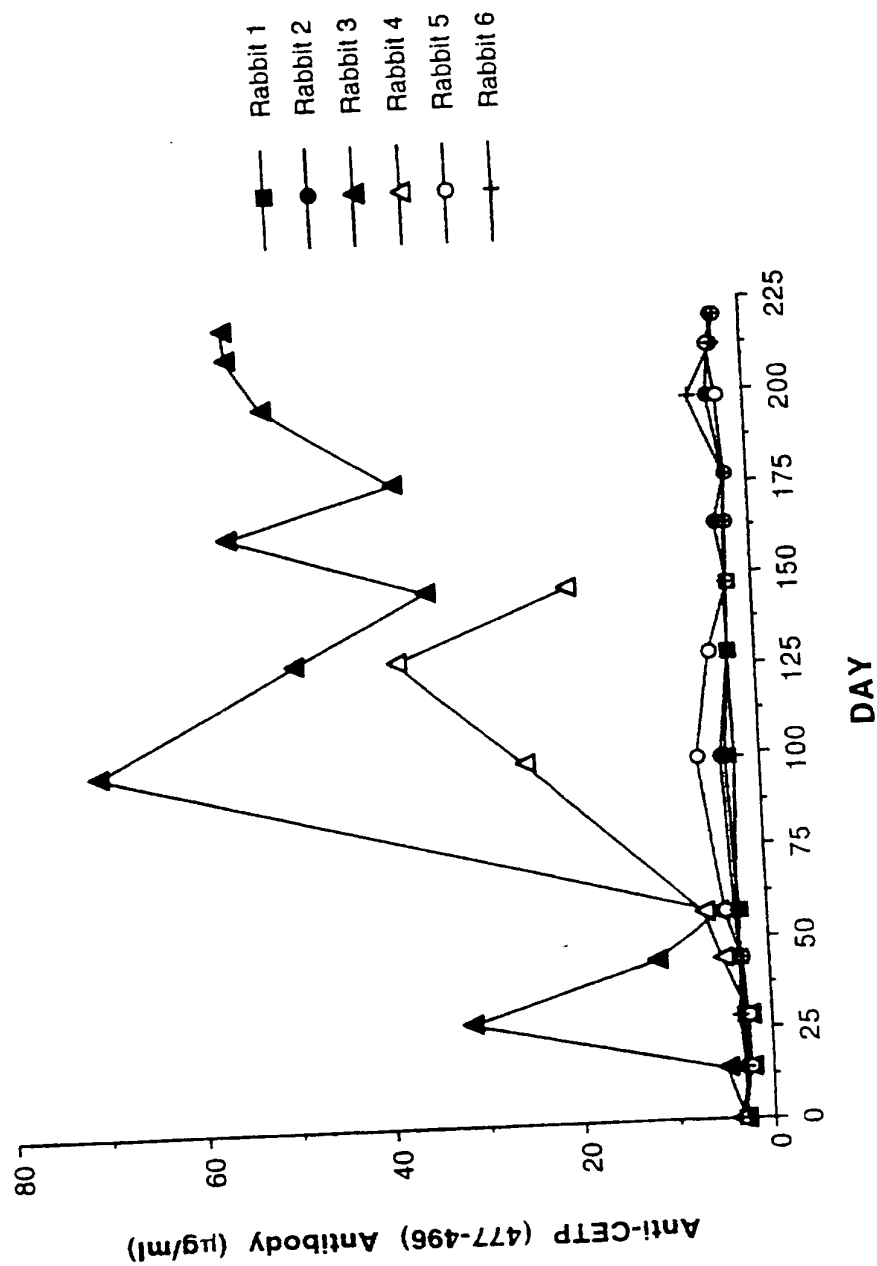
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FIGURE 8

FIGURE 9



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FIGURE 10



INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US 97/07294

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	THOMAS L J ET AL: "A plasmid-based vaccine to elicit autoantibodies to cholesteryl ester transfer protein (CETP) for the prevention-treatment of atherosclerosis" JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY, 99 (1 PART 2). 1997. S187., XP002041706 see abstract 754	1
P,X	WO 96 34888 A (T CELL SCIENCES INC ;RITTERSHAUS CHARLES W (US); THOMAS LAWRENCE J) 7 November 1996 see claims 1-36 --- -/--	17,22. 27,28,31

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- *&* document member of the same patent family

Date of the actual completion of the international search

24 September 1997

Date of mailing of the international search report

07.10.97

Name and mailing address of the ISA

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Fax (+31-70) 340-3016

Authorized officer

Espen, J

INTERNATIONAL SEARCH REPORT

Internat. Application No.
PCT/US 97/07294

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		Relevant to claim No.
Category *	Citation of document, with indication, where appropriate, of the relevant passages	
A	<p>SMITH, A.M. ET AL: "Preparation of an anti-peptide antiserum specific for cholesteryl ester transfer protein (CETP)" MED. SCI. RES., 1993, vol. 21, pages 911-912, XP002041707 see the whole document -----</p>	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 97/07294

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

or

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 97/07294

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remark : Although claims 17-35 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

International Application No
PCT/US 97/07294

Form PCT/ISA 210 (patent family annex) (July 1992)

